(54) Title: DNA-ARMED RIBOZYMES AND MINIZYMES


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Meg                    3' c g c c CA  t a c t t c 5'
                          A    CUG
                          A         A
                          G    AGU
                          eg
                                        eg = teg, heg
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(57) Abstract

The invention describes catalytic nucleic acid based compounds capable of cleaving nucleic acid polymers both in vivo and in vitro. Two embodiments of this invention are compounds with a short stem that does not base pair, a minizyme, and compounds with DNA hybridizing arms and RNA catalytic domain and stem, DNA-armed ribozymes. The compounds of this invention, while nucleotide based may be substituted or modified in the sugar, phosphate, or base. Methods of use and methods of treatment are also described.

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DNA-ARMED RIBOZYMES AND MINIZYMES
Background of the Invention

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Throughout this application various publications are referred to by author and year within brackets. The full references are listed alphabetically after the Experimental Section. The disclosures for these publications in their entireties are hereby incorporated by reference into this application to more fully describe the state of the art to which this invention pertains.

Ribozymes are RNA molecules that can cut or ligate other nucleic-acid molecules (usually RNA) in a catalytic fashion (Cech and Bass, 1986; Altman, et al., 1987). The hammerhead ribozyme is one of the best-known ribozymes. It has been studied extensively in isolated chemical systems (Forster and Symons, 1987; Uhlenbeck, 1987; Haseloff and Gerlach, 1988; Jeffries and Symons, 1989; Koizumi, et al., 1988), and used in gene-control studies in living cells (Cotten and Birnstiel, 1989; Cameron and Jennings, 1989; Sarver, et al., 1990; Saxena and Ackerman, 1990; Sioud and Drlica, 1991; Sioud, et al., 1992). A hammerhead ribozyme as defined by Haseloff and Gerlach (Haseloff and Gerlach, 1988) is shown in Figure 1. It contains two stretches of conserved nucleotides (boxed), a stem-loop structure (bases 18-29) containing helix II, and flanking nucleotides which form double-helices I and III in combination with the substrate.

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The instability of ribozymes in living cells is a major

concern.   One approach taken to protect transcribed
ribozymes from nuclease attack in cells has been to embed
the ribozyme in a larger, folded structure.   Thus,
hammerhead ribozymes have been placed next to the anti-codon
loop in t-RNA^{met} (Cotten and Birnstiel, 1989), the 3'
untranslated region of the luciferase gene (Cameron and
Jennings, 1989), and in a molecule with bacteriophage T7
transcription terminator at its 3' end (Sioud, et al, 1992).
These ribozymes appeared to be more stable than the
corresponding, unprotected ribozymes; however, in the only
comparative study, the stabilized ribozyme did not cleave
more target RNA than the shorter-lived ribozyme, indicating
that the protecting structure may decrease the specific
activity of that ribozyme (Sioud, et al., 1992).

An alternative approach has been to chemically synthesize
ribozymes with ribonucleotides modified at the 2' position.
The modified nucleotides have included 2'-deoxy- 2'fluoro-,
2'-amino-,   2'-O-allyl-   and   2'-O-methyl-ribonucleotides
(Perreault, et al., 1990; Perreault, et al., 1991; Olsen, et
al., 1991; Pieken, et al., 1991; Williams, et al., 1992;
Paolella,   et   al.,   1992).   A   ribozyme   consisting
predominantly   of   2'-O-allyl   ribonucleotides   displayed
greatly   improved   stability   compared   to   an   unmodified
ribozyme in the presence of bovine serum (Paolella, et al.,
1992). Modifications to nucleotides in the hybridizing arms
and/or in helix II of the ribozyme have little effect on
catalytic efficiency (Olsen, et al., 1991; Pieken, et al.,
1991; Williams, et al., 1992; Paolella, et al., 1992); for
example, substitution of the 2'-hydroxyl groups with 2'-O-
allyl   groups   in   all   non-conserved   nucleotides   of   a
hammerhead ribozyme resulted in full retention of activity
(Paolella, et al., 1992).   On the other hand, changing the
2'-substituent in any of the conserved nucleotides of the

ribozyme resulted in a decrease in catalytic activity, the magnitude of which varied greatly depending on the number of changes, the nature of the change, and the particular nucleotides modified (Perreault, et al., 1990; Perreault, et al., 1991; Olsen, et al., 1991; Pieken, et al., 1991; Williams, et al., 1992; Paolella, et al., 1992).

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Summary of the Invention

The invention describes catalytic nucleic acid based compounds capable of cleaving nucleic acid polymers both in vivo and in vitro. Two embodiments of this invention are compounds with a short stem that does not base pair, a minizyme, and compounds with DNA hybridizing arms and RNA catalytic domain and stem, DNA-armed ribozymes. The compounds of this invention, while nucleotide based may be substituted or modified in the sugar, phosphate, or base. Methods of use and methods of treatment are also described.

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Brief Description of the Figures

Fig. 1. Base sequence, schematic representation, and names of ribozyme, minizymes, and their common substrate. Conserved ribonucleotides are drawn in boxes and depicted schematically by thick lines, other ribonucleotides are depicted by uppercase letters and thin lines, and deoxyribonucleotide are depicted by lowercase letters and wavy lines. R, ribozyme containing helix II; M, minizyme not containing helix II; MS, a minizyme and substrate in the same molecule; the first subscript indicates the bases in the connector, and the second subscript indicates whether RNA or DNA nucleotides are in the flanking arms that form helices I and II with the substrate. The ribozyme and minizymes cleave the substrate after the cytidine residue marked by a downward arrow(SEQ ID NO:1-7).

Fig. 2. Plots of percentage of product versus time for cutting of substrate by ribozyme and minizymes, labeled. Reactions were in 50 mM Tris-HCl, pH 8/10 mM $MgCl_2$ at 37°C; substrate concentration was 0.1 μM, and ribozyme and minizyme concentrations were 0.6 μM in all reactions. (a) Effect of reducing size of an all-RNA ribozyme. (b) Effect of replacing RNA nucleotides by DNA in a minizyme of constant length. (Data in a for the ribozyme $R_{4U,RNA}$ are included in b for reference.)

Fig. 3. Observed rate constants ($\square$) for the self-cleavage of the unimolecular minizyme-substrate $MS_{4U,RNA}$ at various concentrations. Reactions were at 37°C in 50 mM Tris-HCl, pH 8/10 mM $MgCl_2$. Solid line represents the expected variation of rate constant with concentration (k α concentration^2), were the reaction bimolecular and dimer-formation rate limiting.

Fig. 4. Mobilities of various substrates, minizymes, and ribozymes, and their complexes and products of cleavage in

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a non-denaturing, 10% polyacrylamide gel at 4°C. The
electrophoresis buffer was 50 mM Tris borate, pH 8/10 mM
$MgCl_2$. Species in each of lanes 4-16 represent the reaction
products for 30 min at 37°C under standard conditions. S,
substrate only in reaction: S' noncleavable substrate; M,
minizyme; R, ribozyme; C, equimolar mix of substrate and
minizyme (or ribozyme); and C', equimolar mix of
noncleavable substrate and minizyme (or ribozyme). The
molecules in set A (lanes 4-6) are an all-RNA, 21-base
synthetic substrate of sequence 5' -AUUUGCGAGUCCACACUGGAG
(SEQ ID NO:8)(from the Krüppel gene of Drosophila
malanogaster) and its cognate, 34-base minizyme based on
$M_{4t,DNA}$; those in set B (lanes 7-11) are the all-RNA, 21-base
substrate described in Fig. 1, a noncleavable substrate of
identical base sequence in which the central ribocytidine
has been replaced by a deoxyribocytidine, and the minizyme
$M_{4t,DNA}$; those in set C (lanes 12-16) are the same cleavage
and noncleavable substrates of set B and ribozyme $R_{4U,RNA}$.
Size markers are labeled in base pairs. Lanes: 1, DNA
double helices formed by a Hpa II restriction digest of
pUC19; 2, 52-bp DNA double helix; and 3, 21-bp double helix
consisting of an all-RNA strand identical to the molecule in
lane 4 and an all-DNA strand of complementary sequence. To
clearly see the bands of interest, the gel above the 190-bp
mark is not shown; there were no bands above this mark in
lanes 2-16.

Fig. 5. Sequences of the ribozymes and substrates used in
this study. Lower case letters are deoxyribonucleotides,
upper case letters are ribonucleotides, and boxed letters
are conserved nucleotides. S21D (SEQ ID NO:9), S13(SEQ ID
NO: 10), Ribozyme 1(SEQ ID NO:11). Ribozyme 2(SEQ ID NO:12)
has the same sequence as ribozyme 1, except that the
underlined letters are all deoxyribonucleotides in ribozyme
1 with U_6, U_7 and U_{10} being replaced by T. Roman numerals

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label double helices. All oligonucleotides used in this
study have a 3'-deoxyribonucleotide.

Fig. 6. Percent product versus time for reaction of
ribozyme 1 ● and ribozyme 2 ■, with substrate S21D at 30°C,
50 mM Tris.Cl, pH 8.0 and 10 mM MgCl$_2$; concentration of
ribozyme was 1.5 μM and substrate, 200 mM. solid lines are
lines of best fit to the data, using the first-order kinetic
equation given in the Methods section.

Fig. 7. The rate of cleavage by the three TAT ribozymes as
a function of substrate concentration is shown. The kinetic
results may be found in Table 4, TATRA (all RNA); TATRB (DNA
hybridizing arms); TATRC (DNA hybridizing arms and DNA helix
II).

Fig. 8. The rate of cleavage of ribozymes and minizyme
targeted against a short 21 bp substrate and a long 428 bp
substrate where the catalyst is in excess.

Fig. 9. The figure shows the relative rates of cleavage of
DNA-armed ribozymes with varying arm lengths targetted
against a variety of target genes.

Fig. 10. The figure shows the relative rates of cleavage of
DNA-armed ribozymes versus all RNA ribozymes against a short
13 bp substrate. GHRA and GHRB are growth hormone RNA
ribozyme and DNA-armed ribozyme respectively.

Fig. 11. The figure shows the structure of three DNA-armed
ribozymes with varying stem length TATRB-H2, TATRB-H4,
TATRB-8(SEQ ID NO:13-15).

Fig. 12. The figure shows the activity of three DNA-armed
ribozymes with varying stem length against a short (21 bp)
TAT substrate at 37°C.

Fig. 13. The figure shows the activity of three DNA-armed
ribozymes with varying stem length against a long (428 bp)
TAT substrate at 37°C.

Fig. 14. The structure of three ribozymes targeted against
a TAT substrate. The first ribozyme has had the phosphates

replaced by phosphothiolates in the stem loop of helix II
(capital letter in italics) and DNA arms (TAT RBPS). The
second ribozyme has RNA arms and a DNA stem (TAT RG) and the
third ribozyme has mixed RNA DNA arms and stem (TAT RH) (SEQ
5   ID NO:16-18).

Fig. 15. The activity of the three mixed DNA RNA ribozymes
against a TAT substrate is shown.

Fig. 16(a) - 16(c). Sequences of the molecules used in this
study. In all of these sequences, upper-case letters
10 represent ribonucleotides, lower-case letters
deoxyribonucleotides, and n represents the number of times
a monomer is repeated in the linker. The target for the two
half-minizymes KrM½A and KrM½B is KrS21 shown at the top;
the target for all other minizymes is S13. The numbering of
15 the conserved nucleotides A9 and G12 follows the scheme of
Hertel et al. Abbreviations: S substrate, M minizyme, pd
phospho-1,3-propanediol, teg tetraethyleneglycol, heg
hexaethyleneglycol. Compounds studied and targets: KrS21,
KrM½A, KrM½B, S13, M(n)pd, Meg, M(n)t, M4t(6x2), M$_{UUCG}$ and
20 GHRB.

Fig. 17 Structures of non-nucleotide linkers. A monomer of
phosphopropanediol (pd) and the full structures of the
linkers tetraethyleneglycol (teg) and hexaethyleneglycol
(heg) are shown. Dashed lines are connections to the 5'O of
25 A9 and the 3'O of G12.

Fig. 18. Log rate constant versus linker length for the
series of minizymes with linkers containing teg, heg and
repeated units of phosphopropanediol (pd), and thymidine
(t). Linker length is defined as the number of atoms
30 between the 3'O of A9 and the 5'O of G12.

Fig. 19. The rigid linker molecule is shown. It was
incorporated into the minizymes utilizing the standard
phosphoramidite chemistry, the purification and workup of
the minizymes was as normal. We have incorporated the

carbazole moiety into two new minizymes: one with the carbazole replacing the linker region (GH M Carb) and the other with the carbazole flanked by phosphopropanediol moieties as the linker region (GH M pcp). The carbazole

5 linkers are incorporated into the same basic minizyme as described in M(n)pd and Meg (Figures 16A and 16B). The kinetics were performed by the same method and using the same 13-mer RNA substrate as described on page 56, line 24 through page 57, line 19.

10 Fig. 20. Structures of two rigid linker compounds, GHM Carb and GHM pcp.

Fig. 21. Cleavage rates of two rigid linker compounds, GHM Carb and GHM pcp. GHM pcp1 and GHM pcp2 show data for two independent experiments for the compound GHM pcp.

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Detailed Description of the Invention

The ribozyme which has been stabilized against intracellular
degradation needs to be reasonably active. In the course of
our work on reducing the size and RNA-content of the
5   hammerhead ribozyme, we produced a minimized ribozyme in
which the stem-loop of helix II was replaced by four
ribonucleotides (McCall, et al., 1992). This 'minizyme' was
less active, at cleaving a synthetic substrate of 21
nucleotides, than its parent ribozyme which contained a
10 helix II. However, the cleavage activity of the minizyme
increased unexpectedly, when the RNA nucleotides in the
hybridizing arms of the minizyme were replaced by DNA
(McCall, et al., 1992). Further, minizymes were more active
than ribozymes against a long substrate vide infra.
15

Ribozymes are RNAs capable of catalyzing RNA cleavage
reactions. One simplest and most commonly used are the
hammerhead type ribozymes which contain a conserved
catalytic domain and flanking sequences that hybridize with
20 the substrate RNA (Haseloff et al. PCT International
Publication No WO 89/05852). Hammerhead ribozymes can be
targeted against any RNA sequence that contain an XUX
triplet amenable for cleavage. Several studies have
demonstrated the ability of these ribozymes to cleave a
25 target RNA in vivo and suppress protein expression. Other
classes of ribozymes are tetrahymena IVS (Group I Intron)
(Cech et al. U.S. Patent No. 4,740,463), RNAse P (Altman et
al. PCT International Publication No WO 92/03566), and
hairpin ribozymes (Hampel et al., 1990). Further,
30 hammerhead compounds with DNA arms, DNA stem loops, and RNA
catalytic region have been described subsequent to
applicants effective filing date (Rossi et al., U.S. Patent
No. 5,144,019).

35 One embodiment of the invention is a minizyme, a compound

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having the structure:
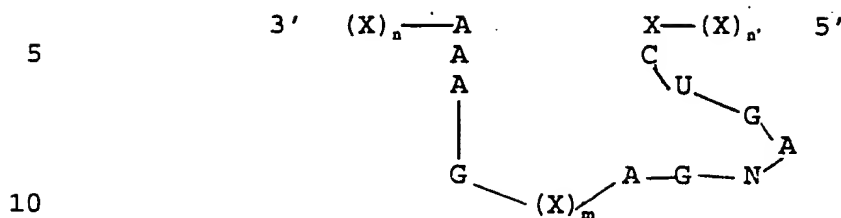
$$3' \quad (X)_n\text{---}A \qquad\qquad X\text{---}(X)_{n'} \quad 5'$$
$$A \qquad\qquad C$$
$$A \qquad\qquad \backslash U$$
$$| \qquad\qquad\qquad\quad \backslash G$$
$$G \qquad\qquad\qquad\qquad\qquad A$$
$$\backslash\quad A\text{---}G\text{---}N$$
$$(X)_m$$

wherein each X is the same or different and represents a ribonucleotide or a deoxyribonucleotide which may be modified or substituted in its sugar, phosphate or base; wherein $(X)_n$ and $(X)_{n'}$ represent oligonucleotides in which n and n' are integers which define the number of nucleotides in the oligonucleotides, such oligonucleotides having predetermined sequences sufficiently complementary to a predefined RNA target sequence to be cleaved to allow hybridization to the RNA target sequence. In one embodiment of the structure above 3' $(X)_n$---A is 3' $(X)_{n-1}$---CA. N may be adenine, guanine, cytosine, or uracil, but preferably cytosine or uracil. In the structure above each solid line represents a chemical linkage providing covalent bonds between the nucleotides located on either side thereof and m represents an integer from 2 to 20. Preferably, the number of nucleotides in the $(X)_m$ is from 2 to 6. In this embodiment of the invention none of the nucleotides $(X)_m$ are Watson-Crick base paired to each other or other nucleotide within the compound.
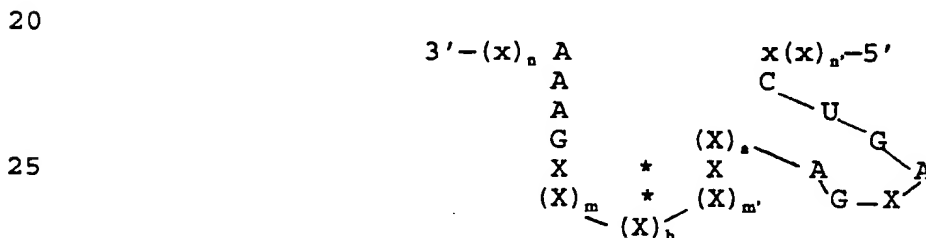
In one embodiment of the invention the compound is entirely composed of RNA. In another embodiment, the hybridizing arms $(X)_n$ and stem $(X)_m$ are composed of DNA.

The absence of base pairing is an advantageous feature of this invention as endonucleases comprising a minimal number of nucleotides may be produced according to standard methods

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described herein.   The applicants have surprisingly
discovered that base pairs between nucleotides in the group
$(X)_m$ are not required to permit the endonuclease of this
invention to cleave its target substrate. Accordingly, the
5   group $(X)_m$ may comprise any number of non base paired
nucleotides, for example, two nucleotides (such as TT), four
nucleotides (such as AAAA, UUUU, TTTT, etc.) or five
nucleotides (such as TTTTT). The nucleotide sequence of the
group $(X)_m$ under these circumstances is not of importance
10   and the number of nucleotides is also not of importance.
The main consideration to take into account is that the
resultant endonuclease is capable of substrate cleavage.
This can be readily measured without undue experimentation
in standard cleavage assays on an appropriate target
15   nucleotide sequences as described herein.

Another embodiment of the invention is a DNA-armed ribozyme,
a compound having the structure:

20

$$
\begin{array}{lll}
3'-(x)_n\ A & & x(x)_{n'}-5' \\
\quad\ \ \ A & & C \\
\quad\ \ \ A & & \diagdown U \\
\quad\ \ \ G & (X)_a & \diagdown G \\
\quad\ \ \ X \quad *\quad X & \diagdown A \quad \diagdown A \\
(X)_m \quad * \quad (X)_{m'} & \diagdown G-X \\
\quad \diagdown (X)_b \diagup &
\end{array}
$$

30   wherein each X represents a ribonucleotide which is the same
or different and may be modified or substituted in its
sugar, phosphate or base; wherein each x (lower case)
represents a deoxyribonucleotide which is the same or
different and may be modified or substituted in its sugar,
35   phosphate or base. Each of A, C, U, and G represents a
ribonucleotide which may be unmodified or modified or
substituted in its sugar, phosphate or base. Each of $(x)_n$
and $(x)_{n'}$ represents an oligodeoxyribonucleotide having a

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predetermined sequence and each of n and n' represents an integer which defines the number of deoxyribonucleotides in the oligonucleotide with the proviso that the sum of n + n' is sufficient to allow the compound to hybridize with the RNA target sequence. In one embodiment, 3' $(x)_n$——A is 3' $(x)_{n-1}$——CA. In some instances it will be desirable to have the hybridizing arms exactly complementary to the target sequence it is not manditory. Each * represents base pairing between the nucleotides located on either side thereof. Preferably, the compound will have two or eight base pairs in the stem. Each solid line represents a chemical linkage providing covalent bonds between the ribonucleotides located on either side thereof. $(X)_a$ may be present or absent in the conserved region. Each of m and m' represents an integer which is greater than or equal to i but prefereably m is 1 or 7. $(X)_b$ represents an oligoribonucleotide and b represents an integer which is greater than or equal to 2. Preferably, $(X)_b$ contains 4 nucleotides.

The invention also encompasses poly minizymes or DNA-armed ribozymes or mixtures thereof e.g. compounds having the structure:

$$3' \quad [-(Y)_r\text{——}Q\text{——}(Y)_s-]_z \quad 5'$$

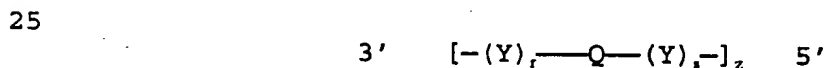wherein Q represents a minizyme or DNA-armed ribozyme as described above which may be the same or different. Each Y represents a spacer, a ribonucleotide or a deoxyribonucleotide which is the same or different and may be modified or substituted in its sugar, phosphate or base. Each of r and s represents an integer which is greater than or equal to 0. z represents an integer from 1 to 100.

Ribonucleotide and deoxyribonucleotide derivatives or

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modifications are well known in the art, and are compatible
with commercially available DNA synthesizers. (See Saenger,
1984, particularly pages 159-200). Nucleotides comprise a
base, sugar and a monophosphate group. Accordingly,
5   nucleotide derivatives, substitutions, or modifications may
be made at the level of the base, sugar, or monophosphate.

A large number of modified bases are found in nature, and a
wide range of modified bases have been synthetically
10   produced (Saenger, 1984; and CRC Handbook of Biochemistry).
Suitable bases would include inosine, 5'- methylcytosine,
5'-bromouracil, xanthine, hypoxanthine and other such bases.
For example, amino groups and ring nitrogens may be
alkylated, such as alkylation of ring nitrogen atoms or
15   carbon atoms such as N^1 and N^7 of guanine and C^5 of cytosine;
substitution of keto by thioketo groups; saturation of
carbon=carbon double bonds, and introduction of a C-glycosyl
link in pseudouridine. Examples of thioketo derivatives are
6-mercaptopurine and 6-mercaptoguanine.
20

Bases may be substituted with various groups, such as
halogen, hydroxy, amine, alkyl, azido, nitro, phenyl and the
like. Bases may be substituted with other chemical species,
such as an amino-acid side chain or linkers which may or may
25   not incorporate other chemical entities, e.g. acidic or
basic groups. For example, guanine (G_3) may be substituted
with tyrosine, and cytosine (C1) or adenine (A11) similarly
substituted with histidine.

30   The sugar moiety of the nucleotide may also be modified
according to well known methods in the art (Saenger, 1984).
This invention embraces various modifications to the sugar
moiety of nucleotides as long as such modifications do not
abolish cleavage activity of the compound. Examples of

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modified sugars include replacement of secondary hydroxyl
groups with halogen, amino or azido groups; 2' -methylation;
conformational variants such as the O_2' -hydroxyl being cis-
oriented   to   the   glycosyl $C_{1'}$ -N   link   to   provide
5   arabinonucleosides, and conformational isomers at carbon $C_{1'}$.
to give α-nucleosides, and the like.   Further, non ribose
sugars may be used such as hexoses such as glucose, pentoses
such as arabinose.

10   The phosphate moiety of nucleosides is also subject to
derivatisation or modifications, which are well known in the
art.   For example, replacement of oxygen with nitrogen,
sulphur   or   carbon   derivatives   to   respectively   give
phosphoramidates, phosphorothioates, phosphodithiolates, and
15   phosphonates.   Substitutions   of   oxygen   with   nitrogen,
sulphur of carbon derivatives may be made in bridging or non
bridging positions.   It has been well established from work
involving antisense oligonucleotides that phosphodiester and
phosphorothioate derivatives may efficiently enter cells
20   (particularly when of short length), possibly due to
association with a cellular receptor.   Methylphosphonates
are probably readily taken up by cells by virtue of their
electrical neutrality.

25   The phosphate moiety may be completely replaced with peptide
nucleic acids (see Hanvey et al., 1992; Nielson, 1991; and
Egholm, 1992).   Other replacements are well-known to those
skilled in the art for example siloxane bridges, carbonate
bridges, acetamidate bridges, carbamate bridges, thioether
30   bridges, etc. (Uhlmann and Peymann, 1990).

In the catalytic region, conserved region (see Figure 1)
nucleotide additions, deletions or replacements may be made
as described above with the proviso that activity is not

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destroyed.   For example, any one of the conserved
nucleotides may be substituted with one or more ribo- and/or
deoxyribonucleotides containing bases such as adenine,
guanine, cytosine, methyl cytosine, uracil, thymine,
5   xanthine, hypoxanthine, inosine, or other methylated bases.

As will be readily appreciated by workers in the field to
which this invention relates, the cleavage of a target RNA
may be readily assessed by various methods well known in the
10   art   or example, see Sambrook et al., 1989). Cleavage may,
for example, be assessed by running the reaction products
(where the substrate is radioactively labelled) on
acrylamide, agarose, or other gel systems, and then
subjecting the gel to autoradiography or other analytical
15   technique to detect cleavage fragments (Sambrook et al.,
1989).

By way of example, blocking groups may be added from
optionally substituted alkyl, optionally substituted phenyl,
20   optionally substituted alkanoyl. Optional substituents may
be selected from C1-C5 alkoxy and the like. Alternatively,
nucleotide analogues such as phosphothioates,
methylphosphonates or phosphoramidates or nucleoside
derivatives (such as α-anomers of the ribose moiety),
25   hexoses such as glucose, non-ribose pentoses such as
arabinose, which are resistant to nuclease attack may be
employed as terminal blocking groups.

Alternatively, non nucleic acid groups which alter the
30   susceptibility of the endonuclease molecule to other
nucleases may be inserted into the 3' and/or 5' end of the
endonuclease. For example, 9-amino-acridine attached to the
endonuclease may act as a terminal blocking group to
generate resistance to nuclease attack on the endonuclease
35   molecules and/or as an intercalating agent to aid

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endonucleolytic activity. It will be readily appreciated that a variety of other chemical groups, e.g. spermine or spermidine could be used in a related manner.

5   One or more ribonucleotides and /or deoxyribonucleotides of the group $(X)_m$ may be replaced, for example, with a linker selected from optionally substituted polyphosphodiester (such as poly(1-phospho-3-propanol)), optionally substituted alkyl, optionally substituted polyamide, optionally

10   substituted glycol, and the like. Optional substituents are well known in the art, and include alkoxy (such as methoxy, ethoxy and propoxy), straight or branch chain lower alkyl (such as C_1-C_5 alkyl), amine, aminoalkyl (such as amino C_1-C5 alkyl), halogen (such as F, Cl and Br) and the like. The

15   nature of optional substituents is not of importance, as long as the resultant endonuclease is capable of substrate cleavage.

Additionally, suitable linkers may comprise polycyclic

20   molecules, such as those containing phenyl or cyclohexyl rings. The linker (L) may be a polyether such as polyphosphopropanediol, polyethyleneglycol, a bifunctional polycyclic molecule such as a bifunctional pentalene, indene, naphthalene, azulene, heptalene, biphenylene, asym-

25   indacene, sym-indacene, acenaphthylene, fluorene, phenalene, phenanthrene, anthracene, fluoranthene, acephenathrylene, aceanthrylene, triphenylene, pyrene, chrysene, naphthacene, thianthrene, isobenzofuran, chromene, xanthene, phenoxathiin, indolizine, isoindole, 3-H-indole, indole, 1-

30   H-indazole, 4-H-quinolizine, isoquinoline, quinoline, phthalazine, naphthyridine, quinoxaline, quinazoline, cinnoline, pteridine, 4-αH-carbazole, carbazole, ß-carboline, phenanthridine, acridine, perimidine, phenanthroline, phenazine, phenolthiazine, phenoxazine,

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which polycyclic compound may be substituted or modified, or a combination of the polyethers and the polycyclic molecules.

5   The polycyclic molecule may be substituted or polysubstituted with C_1-C_5 alkyl, alkenyl, hydroxyalkyl, halogen or haloalkyl group or with O-A or CH_2-O-A, wherein A is H or has the formula CONR'R" wherein R' and R" are the same or different and are hydrogen or a substituted or
10   unsubstituted C_1-C_6 alkyl, aryl, cycloalkyl, or heterocyclic group; or A has the formula -M-NR'R" wherein R' and R" are the same or different and are hydrogen, or a C_1-C_5 alkyl, alkenyl, hydroxyalkyl, or haloalkyl group wherein the halo atom is fluorine, chlorine, bromine, or iodine atom; and -M-
15   is an organic moiety having 1 to 10 carbon atoms and is a branched or straight chain alkyl, aryl, or cycloalkyl group.

In one embodiment, the linker is tetraphosphopropanediol or pentaphosphopropanediol. In the case of polycyclic
20   molecules there will be preferably 18 or more atoms bridging the nucleic acids. More preferably there will be from 30 to 50 atoms bridging, see for Example 5. In another embodiment the linker is a bifunctional carbazole or bifunctional carbazole linked to one or more polyphosphoropropanediol.
25
Such compounds may also comprise suitable functional groups to allow coupling through reactive groups on nucleotides.

Synthetic preparations of mRNA are well known (see Sambrook
30   et al., 1989). Mixed DNA-RNA oligomers with modified base pairs for the ribozyme or minizyme can be prepared by commercially available DNA synthesizers such as those produced by Applied Biosystems, Biosearch, or Milligen (see, e.g., Perrault et al, 1990) for derivatives (Uhlmann, E. and

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Peyman, A., 1990), H-phosphonate monomers see (Agrawal et al
U.S. Patent No. 5, 149,798).

The compounds of this invention may be covalently or non-
5   covalently associated with affinity agents such as proteins,
antibodies, steroids, hormones, lipids, specific nucleic
acid sequences, intercalating molecules (such as acridine
derivatives, for example 9-amino acridine) or the like to
modify binding affinity for a substrate nucleotide sequence
10   or increase affinity for target cells, or localization in
cellular compartments or the like.   For example, the
compounds of the present invention may be associated with
RNA binding peptides or proteins which may assist in
bringing the endonuclease into juxtaposition with a target
15   nucleic acid such that hybridization and cleavage of the
target sequence may take place. Nucleotide sequences may be
incorporated into the 5' and 3' ends of the groups $(X)_n$ and
$(X)_{n'}$ to increase affinity for substrates. Such additional
nucleotide sequences may form triple helices with target
20   sequences (Strobel, et al., 1991) which may enable
interaction   with   intramolecularly   folded   substrate.
Alternatively, modified bases vide supra within the
additional nucleotide sequences may be used that will
associate with either single stranded or duplex DNA
25   generating base pair, triplet, or quadruplet, interactions
with nucleotides in the substrate.

The compounds of the claimed invention may be further
stabilized using methods in the literature for example the
30   use transcription terminators on the 3' end such as the T7
terminator, ρ-independent terminator, cry element (Gelfand
et al. U.S. Patent No. 4,666,848) or the TrpE terminator.
Furthermore, sequences such as the poly(A) addition signal
AATAAA may be added and strategies involving changing the

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length of the 3' non-coding region (see Gillies, U.S. Patent
No. 5,149,635). These techniques can be used to stabilize
RNA in the compound.

5   The invention also embodies methods of production of the RNA
        based compounds described above comprising the steps of: (a)
        ligating into a transfer vector comprised of DNA, RNA or a
        combination thereof a nucleotide sequence corresponding to
        said compound; (b) transcribing the nucleotide sequence of
10   step (a) with RNA polymerase; and (c) recovering the
        compound. The invention also includes transfer vectors,
        bacterial or phage, comprised of RNA or DNA or a combination
        thereof containing a nucleotide sequence which on
        transcription gives rise to the compounds or RNA molecules
15   described above.

        The invention described herein also provides a method of
        cleavage of a specific RNA target sequence which comprises
        reacting a compound (e.g. minizyme, DNA-armed ribozyme, or
20   polymer thereof) with the target sequence so as to thereby
        cleave the specific target sequence. Such target sequences
        may be indigenous to mammals or plants. Preferably, the
        target sequence is in a viral gene. The invention also
        provides a method for the treatment of viral diseases in
25   plants and animals.

        Further, many methods have been developed for introducing
        cloned eukaryotic DNAs into cultured mammalian cells
        (Sambrook et al., 1989):
30   ·   Calcium   phosphate-   or   DEAE-dextran-mediated
                transfection;
        ·   Polybrene;
        ·   Protoplast fusion;
        ·   Electroporation; and
35   ·   Direct microinjection into nuclei.

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Further, the compound described herein may be used in plants
to cleave undesirable mRNA. The appropriate cleavage would
lead to phenotypic changes. Phenotypic changes in plant
cells or plants may include drought resistance, salinity
5   resistance, resistance to fungal, viral or bacterial
infection; modifications of growth characteristics;
sterility; fruit production; flowering; senescence; altering
oil seed metabolic pathways to increase production; and the
like (see Shewmaker et al. U.S. Patent No. 5,107,065). It
10   is evident that one or more RNA involved in determining
phenotype are identified, such RNAs may be inactivated by
cleavage utilizing the endonuclease of this invention and
thus the phenotype of the plant or plant cell altered.
Diseases or infections which may be treated in plants with
15   endonucleases of this invention include fungal infection,
bacterial infections (such as Crown-Gall disease) and
disease associated with plant viral infection.

Phenotypic modifications within animals (including in some
20   applications man)which may be effected by cleaving and thus
inactivating target RNAs associated with phenotype would
include growth characteristics of animals, fertility,
skin/cosmetic modifications, reproductive characteristics,
disease resistance and the like. Myriad applications arise
25   for phenotypic modifications in animals, and plants as
previously mentioned. One or more RNAs associated with a
given endonucleases may be targeted against such RNAs for
their inactivation with consequential phenotypic
modification.
30

Prokaryotic and eukaryotic cell cultures may be
phenotypically modified by treatment with endonucleases of
this invention. For example, bacterial cultures or yeast
cultures involved in production of food components (such as
35   cheese, bread and dairy products) and alcoholic beverage

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production may be treated so as to modify enzyme content, flavor production, cell growth rate, culture conditions and the like. Eukaryotic and prokaryotic cells in culture may, for example be protected from infection or disease

5   associated with mycoplasma infection, phage infection, fungal infection and the like.

The compounds of this invention may also be used to treat diseases or infection in humans, animals, plants, or

10   prokaryotic or eukaryotic cells. The ability to treat disease or infection is based on the fact that the compounds of this invention are capable of cleaving any RNA which contains a suitable cleavage site, such as defined by the generic cleavage site X'UY', where X' and Y' represent any

15   nucleotide (preferably wherein the cleavage site is GUC) as described previously. Most RNAs will contain one or more suitable cleavages sites.

The period of treatment would depend on the particular

20   disease being treated and could be readily determined by a physician. Generally treatment would continue until the disease being treated was ameliorated.

Examples of human and animal disease which may be Herpes

25   Simplex Virus infection (such as targeting cleavage of early gene 4 and 5), psoriasis, cervical preneoplasia, papilloma disease, HIV infection (such as targeting the HIV-1 gag transcript and HIV-1 5'ltr splice site), bacterial and prokaryotic infection, viral infection and neoplastic

30   conditions associated with the production of aberrant RNAs such as occurs in chronic myeloid leukemia.

Further, the targets for the compound may be a viral gene including viral targets such as cytomegalovirus, hepatitis,

35   herpes, HIV, EBV, papilloma virus, rhinovirus, influenza

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virus, varicella-zoster virus, parainfluenza virus, mumps virus, respiratory syncytial virus, adenovirus, measles virus, rubella virus, human parvovirus, poliovirus, rotavirus, echovirus, arbovirus, and human T cell leukemia-
5   lymphoma virus.

An effective amount of a compound of the present invention would generally comprise from about 1 nM to about 1 mM concentration in a dosage form, such as a cream for topical
10   application, a sterile injectable composition, or other composition for parenteral administration. In respect of topical formulations, it is generally preferred that between about 50 μM to about 500 μM endonuclease be employed. Compounds comprising nucleotide derivatives, which
15   derivatives may involve chemically modified groups, such as phosphorothioate or methyl phosphonate derivatives may be active in nanomolar concentrations. Such concentrations may also be employed to avoid toxicity.

20   Therapeutic strategies involving treatment of disease employing compounds of this invention are generally the same as those involved with antisense approaches, such as described in the anti-sense bibliography of (Chrisley, 1991). Particularly, concentrations of compounds utilized,
25   methods and modes of administration, and formulations involved may be the same as those employed for antisense applications.

An "effective amount" as used herein refers to that amount
30   which provides a desired effect in a mammal having a given condition and administration regimen. Compositions comprising effective amounts together with suitable diluents, preservatives, solubilizers, emulsifiers, adjuvants and/or carriers useful for therapy. Such
35   compositions are liquids or lyophilized or otherwise dried

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formulations and include diluents of various buffer content (e.g., Tris-HCL, acetate phosphate), pH and ionic strength, additives such as albumin or gelatin to prevent absorption to surfaces, detergents (e.g., Tween 20, Tween 80, Pluronic
5   F68, bile acid salts), solubilizing agents (e.g., Thimerosal, benzyl alcohol), bulking substances or tonicity modifiers (e.g., lactose, mannitol), covalent attachment of polymers such as polyethylene glycol to the oligonucleotide, complexation with metal ions, or incorporation of the
10 material into or onto particulate preparations of polymeric compounds such as polylactic acid, polyglycolic acid, polyvinyl pyrrolidone, etc. or into liposomes, microemulsions, micelles, unilamellar or multilamellar vesicles, erythrocyte ghosts, or spheroplasts. Such
15 compositions will influence the physical state, solubility, stability, rate of _in_ _vivo_ release, and rate of _in_ _vivo_ clearance of the oligonucleotide. Other ingredients optionally may be added such as antioxidants, e.g., ascorbic acid; low molecular weight (less than about ten residues)
20 polypeptides, i.e., polyarginine or tripeptides; proteins, such as serum albumin, gelatin, or immunoglobulins; amino acids; such as glycine, glutamine acid, aspartic acid, or arginine; chelating agents such as EDTA; and sugar alcohols such as mannitol or sorbitol. Possible sustained release
25 compositions include formulation of lipophilic depots (e.g., fatty acids, waxes, oils). Also comprehended by the invention are particulate compositions coated with polymers (e.g., polyoxamers or polyoxamines) and oligonucleotides coupled to antibodies directed against tissue-specific
30 receptors, ligands or antigens or coupled to ligands of tissue-specific receptors. Further, specific nucleotide sequences may be added to target the oligonucleotides of this invention to the nucleus, cytoplasm or to specific types of cells. Other embodiments of the compositions of
35 the invention incorporate particulate forms protective

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coatings, protease inhibitors or permeation enhancers for various routes of administration, including parenteral, pulmonary, nasal and oral.

5   Suitable topical formulations include gels, creams, solutions, emulsions, carbohydrate polymers, biodegradable matrices thereof; vapors, mists, aerosols, or other inhalants. The oligonucleotides may be encapsulated in a wafer, wax, film or solid carrier, including chewing gums.

10   Permeation enhancers to aid in transport to movement across the epithelial layer are also known in the art and include, but are not limited to, dimethyl sulfoxide and glycols.

       For _in-vitro_ use, the compounds of this invention are
15   generally reacted with a target RNA which contains one or more suitable cleavage sites. Optionally, the target RNA may be purified or substantially purified. The nucleotide sequences $(X)_N$ and $(X)_{N'}$ of the endonuclease of this invention are selected so as to specifically hybridize or

20   form a double-stranded DNA duplex with a target RNA whereafter cleavage takes place. Accordingly, target RNA may be specifically cleaved _in-vitro_ in the presence of other RNAs which themselves would not be cleaved.

25   The compounds may be utilized in a manner similar to restriction endonucleases, that is for the specific cleavage of RNA to facilitate RNA manipulation. All that is required for such manipulations is that the target RNA to be cleaved contains a uracil base and thus a suitable cleavage site.

30

       The compounds of this invention may be utilized in diagnostic procedures, such as the mapping or finger-printing of RNA. Specifically, the compounds of this invention would enable mapping of RNA and may be used to

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detect mutations in RNA sequence. Such procedures may be used in research and may also have forensic and other diagnostic applications.

5   RNA cleavage products _in-vitro_ may be readily detected, for example, by visualization on acrylamide or agarose gels where the amounts of RNA cleaved are sufficiently large for direct visualization after separation and reaction with nucleotide visualization agents, such as ethidium bromide.

10   Alternatively, where the target RNA cleaved is present in small amounts, such as in a sample containing many RNAs, cleavage products may, for example, be detected by using radiolabelled probes for sequence complementary to the target sequence, or amplification techniques such as PCR

15   (Sambrook et al., Supra).

A target RNA for cleavage _in-vitro_ may be derived from any source, and may be of animal, viral, bacterial, plant, synthetic, or other origin. As RNA is common to all known

20   living organisms, this invention may be utilized to cleave any RNA species having a suitable cleavage site as mentioned previously.

This invention is illustrated in the Experimental Detail

25   sections which follow. These sections are set forth to aid in an understanding of the invention but are not intended to, and should not be construed to, limit in any way the invention as set forth in the claims which follow thereafter.

30

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EXPERIMENTAL DETAILS
Experiment 1
Materials and Methods

5   **Solid-Phase Synthesis of Oligonucleotides.** All oligonucleotides were synthesized on an Applied Biosystems 380 and/or 391 synthesizer by using 2-cyanoethylphosphoramidite chemistry. DNA monomers were from Applied Biosystems; RNA monomers, protected at the 2'

10   position with a _tert_-butyldimethylsilyl group, were from Peninsula Laboratories or MilliGen (Bedford, MA). The 3' nucleotide in all molecules is a deoxyribonucleotide. All RNA-containing oligonucleotides, with 5'-trityl groups removed, were processed as follows. The oligonucleotide was

15   cleaved from the column in NH_4OH/ethanol, 3:1, and heated overnight at 55°C. The solutions were evaporated to near dryness, coevaporated several times with H_2O/ethanol, 3:1, and then the amount of material was estimated by measuring UV absorbance. The 2'-group of the sugar was deprotected by

20   treatment overnight with 1 M tetrabutylammonium fluoride in tetrahydrofuran (10μl per OD_{260} unit). The tetrabutylammonium ions were removed by passing the oligonucleotide solution twice through a Dowex 50X8-200 cation-exchange column in the Na^+ from; eluate volume was

25   reduced by extraction with 2-butanol, and the oligonucleotide was precipitated with sodium acetate and ethanol. The oligonucleotide was then purified by electrophoresis on a 10-20% (depending on length) polyacrylamide gel containing 7 M urea. The band of

30   interest was visualized by UV shadowing or ethidium bromide staining, excised, and soaked in several changes of water over 24 hr. The supernatant was removed from the gel slices, concentrated with 2-butanol, and extracted with phenol/chloroform and with ether. The oligonucleotide was

then precipitated with sodium acetate and ethanol, washed
with cold 80% ethanol, redissolved in 10 mM Tris·HCl, pH
8.0/0.2 mM EDTA, quantified by UV spectroscopy, and frozen.
The oligonucleotides were phosphorylated on their 5'-ends by
5   using [γ-^{32}P]ATP and T4 polynucleotide kinase (Bresatec,
Adelaide, Australia) under standard conditions, except that
several units of ribonuclease inhibitor (RNasin, Promega)
were added to the reaction mixture. The phosphorylation
reaction was stopped with EDTA; the oligonucleotide solution
10 was extracted with phenol/chloroform and ether and
concentrated with 2-butanol.   The 5'-end labeled
oligonucleotide was precipitated twice, first from 2.5 M
ammonium acetate and ethanol and secondly from 0.3 M sodium
acetate and ethanol. The pellet was washed with cold 80%
15 ethanol and then dissolved in 10 mM Tris·HCl, pH 8/0.2 mM
EDTA to make a stock solution with nominal concentration of
1 μM. The exact concentration of the stock solution was
determined in the following way. All supernatants from the
two precipitation steps and the final ethanol were pooled,
20 concentrated under vacuum, and loaded on denaturing
polyacrylamide gel alongside a known, small fraction of the
stock solution.   After electrophoresis, gel slices
containing the stock and the lost oligonucleotide were
excised, and the amount of material they contained was
25 quantified by Cerenkov counting. These data were used to
determine the exact concentration of the 5'-end-labeled
oligonucleotide solution.

Transcription of Oligonucleotides.   One of the
30 oligoribonucleotides used in this study (MS$_{4U,RNA}$) was
transcribed from a DNA oligonucleotide containing a
truncated T3 promoter. The DNA duplex was generated by
extension with T7 DNA polymerase (Sequenase, United States
Biochemical) after hybridizing two oligonucleotides with

sequences as follows: T3 primer: 5' AAT TAA CCC TCA CTA
(SEQ ID NO:19) and MS template: 5'-ACC TGC GGG TTT CAA AAT
CAT CAG ATG AAG TGT CCG AAG ACA CTT CAT GAC CCG CAG GTA AAC
CTT TAG TGA GGG TTA AAT (SEQ ID NO:20).

Another RNA molecule, the 173-nucleotide (nt)
chloramphenicol acetyltransferase (CAT) substrate, was
transcribed with T7 RNA polymerase from a _Pvu_ II digest of
pGEM3 containing the _Bam_HI CAT fragment from pCM4 as
described (Haseloff and Gerlach, 1988). Transcripts were
made with the Stratagene transcription kits; a typical
reaction in a 30-μl vol contained 5 pmol of template; 2
units of T3 RNA polymerase; 500 μM each of rATP, rGTP, and
rCTP; 100 μM rUTP; 10 pmol of [α-^{32}P]rUTP (17 μCi; 1 Ci=37
GBq); 10 mM dithiothreitol; CAT RNA was stopped after 30
min, but MS$_{4U,RNA}$ was stopped after only 5 min to minimize
self-cleavage of the transcribed molecule. The mixtures
were extracted with phenol/chloroform; RNAs were
precipitated with sodium acetate and ethanol, and the
pellets were dissolved in sterile water. Concentration of
the solution was determined by knowing the number of
uridines in the sequence, the specific activity of the [α-
^{32}P]rUTP used, and the activity of an aliquot of the
transcript solution.

Cleavage Reactions. Standard conditions for the cleavage
reactions are as follows. Reactions were at 37°C in a 30-μl
vol of 10 mM MgCl$_2$/50 mM Tris·HCl, pH 8.0; the
ribo/minizymes and substrate (labeled on the 5' end with ^{32}P)
were placed quickly on ice before mixing, adding MgCl$_2$, and
incubating. At appropriate times, a 3-μl aliquot was
removed from the reaction and added to 6 μl of quenching
solution containing 80% formamide, 20 mM EDTA, and dye. The
quenched samples were then analyzed by electrophoresis on

15% polyacrylamide gels containing 7 M urea as a denaturant. The substrate and product of cleavage were visualized by autoradiography, and gel slices corresponding to their positions were excised and quantified by Cerenkov counting.

5   The ratio of [product] to ([substrate] plus [product]) was plotted versus time as shown. Rate constants were calculated by fitting, by a Newton-Raphson iterative procedure, data for percentage of product formed (%P) as a function of time (t) to an equation of the form %P = %P$_{\infty}$ is

10   percentage of product at infinite time, C is the difference between percentage of product at t = ∞ and t = 0, and k is the first-order rate constant. A listing of the program, which runs on MS-DOS computers, is available on request.

15   **Nondenaturing Gels.** The molecular species in a mixture that initially contained equimolar amounts (0.1 μM) of 5'-end-labeled substrate and ribozyme or minizyme and in which conditions for the cleavage reaction were as described above were analyzed under nondenaturing conditions by

20   electrophoresis on 10% polyacrylamide gels kept at 4°C. After a 30-min reaction at 37°C, samples were mixed with an equal volume of 30% sucrose/0.1% dye and stored at -20°C. The polyacrylamide gels were preelectrophoresed for 2 hr next day after replacing the buffer with fresh solution (16,

25   17). Then samples were loaded and electrophoresed for 8 hr at constant voltage (400 V). In addition, the same samples were electrophoresed for 6 hr at 400 V on another 10% polyacrylamide gel, which was buffered by 90 mM Tris borate, pH 8/2 mM EDTA containing no Mg^{2+}. The components of

30   several bands in the nondenaturing gels were determined by excising the bands, eluting the molecules in formamide, and separating them by electrophoresis on a denaturing, polyacrylamide gel; the 3'-product of cleavage of a 5'-end-labeled substrate is unobservable by this method.

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Results and Discussion

The hammerhead ribozyme (Haseloff and Gerlach, 1988) is shown in Fig. 1 at top; its sequence (the two conserved
5 regions are drawn in boxes) is at left, and a schematic drawing is at right. We call this ribozyme $R_{4U, RNA}$ and use it as a reference in assessing cleavage activities of other molecules. The RNA substrate for this ribozyme is also shown in Fig. 1 (top), where the cut site follows the
10 cytidine residue marked by a downward arrow. The ribozyme and substrate together are designed to form two double helices each containing 10 base pairs (bp) on either side of the central cytidine. When the substrate is radioactively labeled at its 5' end, the cleavage reaction can be
15 monitored easily by electrophoresing the reaction mixture on a denaturing polyacrylamide gel.

To see how much a ribozyme could be reduced in size without complete loss of activity, we synthesized chemically, in
20 small amounts, a series of RNA molecules that contained the conserved bases but omitted as many of the nonconserved bases as possible. A drastic reduction of the lower domain from 22 to 10 nt, shown in Fig. 1 as $M_{0,RNA}$, was unsuccessful in that it eliminated all RNA-cutting activity (Fig. 2a).
25 But a less drastic reduction from 22 to 14 nt, shown in Fig. 1 as $M_{4U,RNA}$, preserved much cutting activity. The diminished ribozyme, $M_{4U,RNA}$, which lacks a helix II, is less active than the full-size ribozyme $R_{4U,RNA}$ (Fig. 2a, Table 1), but its measurable activity indicates that helix II (previously
30 thought essential) is dispensable to the cleavage reaction. We defined a ribozyme that does not contain helix II as a "minizyme."

To see how many deoxyribonucleotides could be introduced

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into the minizyme ($M_{4U,RNA}$) without activity loss, we
synthesized a series of molecules in which some or all of
the nonconserved RNA nucleotides were replaced by DNA
nucleotides. First, most of the ribonucleotides in the
5   minizyme that contribute to forming double helices I and III
were replaced with DNA (wavy lines in $M_{4U,DNA}$, Fig 1).
Unexpectedly, this minizyme with DNA arms cut the substrate
to a greater extent than the all-RNA minizyme (Fig. 2b,
Table 1). To continue the trend, all nucleotides connecting
10 the two regions of conserved bases were replaced with DNA
(wavy lines in $M_{4L,DNA}$, Fig. 1), and the RNA-cutting activity
of this minizyme equaled that of the reference (Fig. 2b,
table 1). Thus, we have made a diminutive ribozyme that
contains just 12 RNA nt, with 4 DNA nt connecting the two
15 regions of conserved RNA bases, and all DNA elsewhere in the
helix-forming arms; and this small, DNA-rich molecule
cleaves the substrate as well as the full-size, all-RNA
ribozyme, at least for the particular substrate studied
here. We have not attempted to replace any of the conserved
20 ribonucleotides with DNA in the minizymes because such
replacements in an all-RNA hammerhead ribozyme decrease its
activity significantly (Perreault, et al., 1990, Perreault,
et al., 1991). The increase in cleavage activity seen upon
replacing ribonucleotides with deoxyribonucleotides in the
25 minizyme probably arises from a slight alteration to the
structure of the catalytic core of the minizyme-substrate
complex; a different structure must be adopted by a
ribozyme-substrate complex in which ribonucleotides are
replaced by deoxyribonucleotides in the substrate, and for
30 which an activity decrease is seen (Yang, et al., 1990).

To make the minizyme even smaller, the number of nucleotides
in the helix-forming (hybridizing) arms may be reduced. We
experimented with minizyme $M_{4L,DNA}$ and found that the number

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of base pairs in helices I and III can be reduced from 10 to 4, in both double helices, with maintenance of cleavage activity. Thus a minizyme can be at least as small as 22 nt.

Effect of Length and Sequence of the Connector. Within the small DNA-rich minizyme, the only nucleotides that can be varied now are those connecting the two regions of conserved RNA bases; all other nonconserved nucleotides are in the helix-forming arms and, hence, are determined by the base sequence of the target. Therefore, to find the optimal composition of the connector, new minizymes were designed based on the most active minizyme, $M_{4t,DNA}$. To find the optimal length, we compared the cleavage activities of four minizymes containing the DNA nucleotides 2T, 3T, 4T, 5T, or 6T in their connectors. Observed rate constants and $\%P_\infty$ (Table 1) show that the 5T minizyme is marginally better than the 4T minizyme with shorter connectors. Presumably, a connector with <4T restricts the minizyme in adopting the active conformation of cleavage. To look at the effect of the 4-nt sequence, we compared cleavage activities of four minizymes containing the RNA nucleotides 4U, or the DNA nucleotides 4A, 4T, or 5'-TTCT in their connectors. Observed rate constants and $\%P_\infty$ (Table 1) indicate that, of this series, connectors of 4T and TTCT provide for the greatest activity. The connectors 4T and TTCT probably are sufficiently flexible to allow the conserved RNA nucleotides to adopt the active conformation for cleavage, whereas the connectors 4A and 4U are more rigid in structure, with possible stacking of adenine bases in 4A and interactions involving 2'-hydroxyl groups in 4U (Saenger, 1984). Goodchild and Kohli (Goodchild and Kohli, 1991) also have reduced the number of nucleotides joining the two stretches of conserved RNA bases in a hammerhead ribozyme, although

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they kept bases that potentially could form Watson-Crick pairs (and, potentially, a ribozyme dimer). One of their all-RNA molecules, which has the sequence 5'-GGCC in the equivalent of our connector, cleaves its substrate very

5 slowly. A second molecule, which has 5' -GGCGCC in the connector, cleaves the same substrate with higher initial rates. Because the structure of these two connectors would be relatively rigid, due to stacking of guanine bases (McCall et al. 1985) and the possible formation of a GC base

10 pair, a connector >4 nt is probably necessary to allow their enzyme to adopt the active conformation. These initial results suggest that further improvement of the cleavage activity of minizymes may be possible.

15 Table 1. Kinetic data for cleavage by various minizymes

Ribozyme/minizyme	Rate constant, min^{-1}	%P$_\infty$
R$_{4U,RNA}$	0.15	59
M$_{4U,RNA}$	0.05	31
M$_{4U,DNA}$ (4U)	0.05	54
M$_{4t,DNA}$ (4T)	0.16 (0.03)	62(5)
M$_{2t,DNA}$ (2T)	<0.001	--
M$_{3t,DNA}$ (3T)	0.014	65
M$_{5t,DNA}$ (5T)	0.23	71
M$_{6t,DNA}$ (6T)	0.23	62
M$_{4a,DNA}$ (4A)	0.02	64
M$_{ttct,DNA}$ (TTCT)	0.21	76
M$_{4t,Krüp-DNA}$ + Krüppel substrate	0.23	76
M$_{4t,CAT-DNA}$ + short CAT substrate	0.03	70
M$_{4t,CAT-DNA}$ + long CAT substrate	0.02	94

35 Reactions were in 50 mM Tris-HCl, pH 8/10 mM MgCl$_2$ at 37°C; substrate concentration was 0.1 μM and ribozyme and minizyme concentrations were 0.6 μM. Except where indicated otherwise, the substrate is that shown at top of Fig. 1. Minizyme M$_{4t,DNA}$ was synthesized several times, and its

40 kinetics of cleavage was determined independently on four separate occasions; SDs of the mean rate constant and mean percentage of product at infinite time (%P$_\infty$) are in parentheses.

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Minizymes are Active as Monomers. We considered whether some form of helix II might arise from the association of bases in the connectors of two minizyme molecules and whether such a minizyme dimer was the active species. A precedent exists for this: Forster et al. (Forster, et al., 1988) have proposed that self-cleavage of RNA occurs for avocado sunblotch viroid and in cleavage of RNA from the newt by the combination of two different hammerhead domains to form active dimers. However, results from the following three experiments indicate that the minizyme acts as a monomer. (i) For $M_{4t,DNA}$, the putative helix II would be formed by T-T base pairs, which are relatively unstable. A more stable helix II, consisting of T-A base pairs, might be formed by mixing $M_{4t,DNA}$ with $M_{4a,DNA}$; and, were dimerization required for cleavage, the mixed minizyme-dimer should be more active than the dimers formed by either component alone. Cleavage of substrate (0.4 μM) by $M_{4t,DNA}$ (0.4 μM), by $M_{4a,DNA}$ (0.4 μM), and by $M_{4t,DNA}$ mixed with $M_{4a,DNA}$ (0.2 μM each) was followed under standard conditions. The observed rate constants for the reactions (0.14 min^{-1} for $M_{4t,DNA}$, 0.02 min^{-1} for $M_{4a,DNA}$, and 0.04 min^{-1} for $M_{4t,DNA}$ mixed with $M_{4a,DNA}$) indicate no synergy between the two minizymes in cleaving substrate. (ii) The transcribed oligonucleotide $MS_{4U,RNA}$, which has a minizyme and attached substrate (see Fig. 1, bottom), shows little change in the rate constant for cleavage ($\approx$0.1 min^{-1}) over a concentration range varying 25,000-fold from 1.3 μM to 55 pM (data points in Fig. 3). Were the concentration of $MS_{4U,RNA}$ in the cleavage reactions significantly less than the association constant for the putative dimerization, the rate of cleavage by the dimer would be expected to depend on the square of the concentration (solid line in Fig. 3); the data do not following this relation ship. (iii) Complexes of a ribozyme and two different minizymes with various cleavable and noncleavable substrates were analyzed by electrophoresis

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on a 10% polyacrylamide gel under non-denaturing conditions (Fig. 1). A single complex is formed between the minizyme $M_{4L,DNA}$ and its noncleavable substrate, as revealed by the strong band in lane 11, set B, of Fig. 4. This complex

5   moves in the gel as a species of ~31bp, slightly ahead of the 34-bp DNA marker i lane 1. [This estimate of size in bp of a DNA-RNA complex probably represent an upper limit because mobilities of double-stranded RNA molecules and probably, of double-stranded RNA-DNA molecules in

10   nondenaturing gels are 10-20% lower than the corresponding mobilities of duplex DNA (Gast et al., 1991)]. A complex formed by one minizyme (34 nt) and one substrate molecule (21 nt) would contain 55 nt or formally, 27.5 bp. Therefore, in this complex, there is just one minizyme

15   molecule. The adjacent lane 10 contains the same minizyme $M_{4L,DNA}$ with its cleavable substrate. The strong band at ~31 bp in lane 10 was shown to consist of minizyme, uncleaved bp in lane 10 was shown to consist of minizyme, uncleaved substrate, and the 5'-product of cleavage. Other bands

20   lower down lane 10 were shown to contain the minizyme plus the 5'-product, the minizyme plus (presumably) the 3'-product and, at the gel bottom, the 5'-product alone. There are no species of molecular mass > ~31 bp in either lane 10 or 11. Similar results were obtained for a different, 21-

25   nt, cleavable substrate and it respective, 34-nt minizyme (compare lane 6, set A, with lane 10 set B, of Fig. 4), indicating that these observations probably are general for minizymes. Therefore, all available evidence is consistent with minizymes acting as monomers.

30

Other pertinent information can be drawn from Fig. 4 (i) A single complex, which moves in the gel as an ~42-bp species, is formed by the ribozyme $R_{4U,RNA}$ (42 nt) and its noncleavable substrate (21 nt), as shown by the strong band in lane 16,

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set C, of Fig. 4, A 1:1 complex of these molecules would
contain 63 nt or, formally, 31.5 bp.   The same ~42 bp
species is present in lane 15 of Fig. 4; it was shown to
consist of ribozyme, uncleaved substrate, and the 5'product
5   of cleavage, analogous to the minizyme-substrate complexes
in lanes 6 and 10.   In addition, lane 15 reveals that some
higher-polymeric species are present in very small amounts
in the reaction mixture.   All these species, which appear
reproducible, were shown to contain ribozyme, uncleaved
10   substrate, and product of cleavage.   These higher molecular-
mass species depend on Mg^{2+} for stability because they are
absent in 10% polyacrylamide gels buffered by 90 mM Tris
borate/2 mM EDTA.   Therefore, from the experiments on gel
mobility, we conclude that this full-size ribozyme acts
15   predominantly as a monomer in forming a complex with is
substrate.   (ii) The presence of uncleaved substrate
complexed with minizyme and ribozyme in the reaction
mixtures suggests that a proportion of the complexes might
not be in the active conformation for cleavage; this would
20   account for the observed extents of cleavage being <100% in
Fig. 2 and Table 1.   (iii) The minizyme-substrate complex
migrates in the gel as an ~31 bp species, which is only
slightly slower than would be expected were it a regular
double helix of 27.5 bp. Because the mobility of a molecule
25   in a gel is determined by its convex volume (the volume
occupied by an imaginary, convex hull enveloping the
molecule) (Calladine, et al., 1991), this suggests that
helices I and III in the complex are approximately parallel
and that the conserved nucleotides protrude very little
30   beyond the surface of these helices.

Cleavage of Other Substrates. To see whether minizymes were
active generally against other RNA molecules, we synthesized
two more minizymes based on $M_{4L\text{DNA}}$ but with appropriate

changes to the sequence of the flanking arms.   The new
substrates were synthetic oligonucleotides of 21 bases; one
of these is the Krüppel substrate described in set A of Fig.
4, and the other, of sequence 5'-GCAUUUCAGUCAGUUGCUCAA, (SEQ

5 ID NO:21) is part of the gene for CAT.   These short
substrates were cleaved by their respective minizymes at the
expected sites, as judged by counting bands on a denaturing
polyacrylamide gel that had the products of the cleavage
reactions   directly   beside   the   products   of   alkaline

10 hydrolysis of the respective substrates.   To test the
activity of minizymes against a longer RNA substrate, we
made a 173-nt CAT transcript (Haseloff and Gerlach, 1988)
containing the sequence of the shorter substrate, in which
the expected cleavage site was 139 nt from the 5' end.   The

15 minizyme cleaved the transcript at the expected site, as
judged by sizes of the cleavage products.   Rate constants
and %P$_\infty$ measured for these reactions under standard
conditions are given in Table 1.

20 A   further   series   of   growth   hormone   RNA   targeted
endonucleases   were   synthesized   based   on   the   M4t,DNA
construct   and   having   different   types   of   nucleotides
connectors.   For example, MttPDt, DNA where PD refers to
1,3-propanediol which was used in place of a nucleotide.
25

Kruppel RNA:

The endonuclease M4t.DNA, Kr1079 (comprising 34 nucleotides
and having flanking sequences of DNA designed to hybridize
to the Kruppel target RNA) was tested against a short

30 synthetic RNA substrate of 21 nucleotides and a RNA
substrate of approximately 1.9Kb, both containing the same
cleavage site.

The Kr RNA transcript was prepared by inserting cDNA

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encoding the Kr transcript into a plasmid containing the T7RNA polymerase promoter. The Kr transcript was then transcribed with T7-polymerase.

5   The synthetic 21 mer was chemically synthesized and contained the same cleavage site as the longer RNA transcript. The 21 mer comprised the following sequence:

    A U U U G C G A G U C* C A C A C U G G A G

where C* is a ribonucleotide.

10

In-vivo testing of activity of an anti-Kruppel endonuclease could be accomplished by microinjection of Drosophila embryos prior to the stage of syncytial blastoderm, in order to inactivate the 2.3Kb RNA Kruppel transcript. Embryos

15   (cuticlised embryos) can be assayed for abberant segmental pattern one to two days after egg laying.

Platelet Derived Growth Factor (PDGF):
The endonuclease Mttct,DNA, PDGF (a 30 mer, having flanking

20   DNA sequences designed to hybridize to the PDGF target RNA) was reacted with a 666 base RNA transcript corresponding to exons 2 and 6 of the PDGF A gene from human. A 666 base pair RNA transcript was transcribed in-vitro from a PDGF gene fragment using T3 polymerase.

25

In summary, cleavage activity of a ribozyme of the Haseloff-Gerlach type can be maintained despite size reduction and replacement of many RNA nucleotides by DNA. One of the three conserved double helices, helix II, is dispensable to

30   formation of the active structure. The minimized ribozyme, or minizyme, is active as a monomer. We think that these minizymes will be useful in future structural and functional studies of catalytic RNA. They should also prove useful in gene-control studies in living cells (Cotten and Birnstiel,

35   1989; Cameron and Jennings, 1989; Sarver, et al., 1989;

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Saxena and Ackerman, 1990), where the DNA component should make them more resistant to RNase.

Experiment 2

5   **MATERIALS AND METHODS**

All oligonucleotides were prepared by solid-phase methods using 2-silyl-protected phosphoramidites (Milligen) for RNA (benzoyl-protected A, G and C) and phosphoramidite monomers for DNA (Applied Biosystems). The oligonucleotides were

10   deprotected as described above (see McCall, et al., 1992), and purified by electrophoresis on 10 - 20% polyacrylamide gels (depending on the length of the oligonucleotide) containing 7M urea, also as described (McCall et al., 1992). The purity of each oligonucleotide was checked by labelling

15   its 5'-terminus with ^{32}P phosphate using T4 polynucleotide kinase molecules by autoradiography. The concentrations of the purified oligonucleotides were determined by UV spectroscopy using the following molar extinction coefficients for the various nucleotides at 260 nm; A, 15.4

20   X 10^3; G, 11.7 X 10^3; C, 7.3 X 10^3; T, 8.8 X 10^3; U, 10.0 X 10^3. All oligonucleotides were stored in either water or 10 mM Tris·Cl, pH 8.0, 0.2 mM EDTA at -20°C.

Enzyme kinetic experiments were conducted in 50 mM Tris.Cl,

25   pH 8.0, 10 mM MgCl$_2$, at 30°C. The concentration of the all-RNA substrate (S13, Figure 5) ranged from 10 to 200 nM, and the concentration of both ribozyme 2 (Figure 5) was 0.77 nM. Initial-rate measurements were made up to 15% cleavage of the substrate. Neither ribozyme nor substrate were heat-

30   treated before initiating the reaction by adding the substrate. Reactions in 20-40 23μl volumes were performed in 96-well polypropylene tissue-culture trays, as these were found to absorb less ribozyme or substrate than either siliconized or autoclaved Eppendorf tubes. Samples of 2-3

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μL were removed at given times and quenched with 2 volumes
of 80% formamide containing 20 mM EDTA and dye. Samples
were analyzed by electrophoresis on 15% denaturing,
polyacrylamide gels, followed by autoradiography and
5   excision of the bands corresponding to the substrate and 5'
cleavage product. The amounts of radioactivity in the bands
were quantitated by Cerenkov counting. Enzyme kinetic data
were analyzed by Eadie-Hofstee plots to yield K_m and V_{max}.
It was found that K_m and V_{max} from individual experiments
10   varied by up to a factor of 2, and therefore the initial
rates at each concentration, from at least 4 independent
experiments, were averaged and plotted in the form of rate
versus rate/[substrate] to yield the kinetic parameters in
Table 2.
15

Experiments with ribozyme in excess of the substrate S13
were also conducted in 50 mM Tris.Cl, 10 mM $MgCl_2$, at 30°C:
the concentrations of the ribozymes were 1.5 μM and the
substrate was 500 nM. The ribozyme and substrate were
20   heated together to 75°C for 3 minutes, then allowed to cool
to 30°C for 1-2 minutes before initiating the reaction by
adding the magnesium-containing buffer. The kinetic
parameters were obtained by fitting the data for percentage
of product formed versus time to the equation:
25

$$P_t = P_\infty - (exp(-kt)P_\Delta)$$

where P_t is the percentage of product at any given time, P_∞
is the percentage of product at t = ∞, k is the first-order
30   rate constant for the reaction, t is the time, and P_∞ is the
difference between the percentage of product at t = ∞ and t
= 0. This is a conventional first-order kinetic equation
from which k, P_∞ and P_Δ are determined by least-squares
fitting of the data. The quoted rate constants and P_∞

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values in Table II are the mean (± SD) for at least 2
independent experiments.   In the time-scale of these
experiments, the reactions do not proceed to 100%.

5   Kinetics of cleavage of the DNA substrate containing a
     ribocytodine at the cleavage site (S21D, Figure 1) were
     conducted under conditions identical to those described
     above for the experiments with S13, except that the
     concentrations of the ribozymes were 1.5 μM and the
10   substrate was 200 mM.

     Stability of the ribozymes in serum was determined by
     incubating the ribozymes, labelled at the 5' end with ^{32}P
     phosphate, in various concentrations (to 0.01%) of fetal
15   calf serum (Cytosystems).   Samples were removed at various
     times EDTA and dye, and then quickly frozen on dry ice. The
     solutions were thawed immediately prior to loading on to a
     10% denaturing (7 M urea) polyacrylamide gel for analysis.
     Percentage ribozyme remaining at these times was determined
20   by excising gel fragments corresponding to the position of
     the full-length ribozyme and quantitating by Cerenkov
     counting.

     RESULTS AND DISCUSSION
25

     Cleavage of the RNA substrate

     Ribozyme 1 is an all-RNA hammerhead ribozyme with the
     sequence shown in Figure 5.   Ribozyme 2 is an analogous
30   ribozyme in which the non-conserved nucleotides in the
     hybridizing arms, underlined in Figure 5, are DNA, and all
     other nucleotides are RNA. As described in Experiment 1, we
     have observed that when helix II was removed from ribozyme
     1 and replace by a 4-nucleotide linker, a very large

proportion of the resultant loss in activity could be regained by placing DNA in the hybridizing arms of the minimized ribozyme (McCall et al., 1992). Therefore, we synthesized ribozyme 2 in order to see if an increase in
5   activity also could be achieved by placing DNA in the hybridizing arms of a normal hammerhead ribozyme.

The cleavage reactions were analyzed according to Scheme 1, using Michaelis-Menton equations. In the scheme, S is
10   substrate, R is ribozyme, and P_1 and P_2 are the cleavage products. The scheme is a simplification of the real situation since (i) it does not allow for alternate conformations of any of the participants and (ii) the dissociation of the products of the reaction is undoubtedly
15   a multistep process. However, it is acceptable to approximate the product dissociation to a single step since one of the products, by virtue of its G/C content, is expected to dissociate from the ribozyme much more slowly than the other; and the question of alternate conformations
20   will be addressed as it arises.

$$S+R \underset{k_{-1}}{\overset{k_1}{\rightleftarrows}} RS \underset{k_{-2}}{\overset{k_2}{\rightleftarrows}} RP_1P_2 \underset{k_{-3}}{\overset{k_3}{\rightleftarrows}} R + P_1 + P_2$$

25
                              Scheme 1
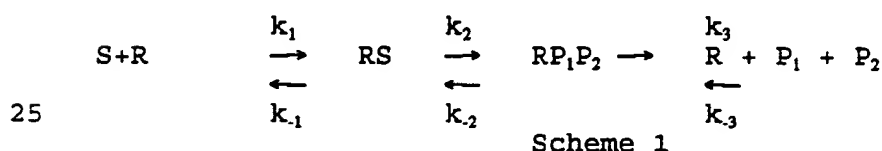
Table 2 <u>vide</u> <u>infra</u> shows the values of the catalytic constant, K_{cat} ($k_{cat} = V_{max}/[ribozyme]$) and the Michaelis
30   constant, K_m, for ribozymes 1 and 2 in reactions, with an RNA substrate of 13 nucleotides at 30°C, k_{cat} for ribozyme 2, 8, 9 min^{-1}, is 20-fold higher than for ribozyme 1. The k_m's for the two ribozymes differ slightly, with ribozyme 1 having the lower value, 38 nM. The ratio k_{cat}/k_m, which is
35   often used as a measure of the relative efficiency of enzymatic activity, is 10 min^{-1} μM^{-1} for ribozyme 1 and 150

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min^{-1} μM^{-1} for ribozyme 2.

The catalytic constant of 8.9 min for the DNA-armed ribozyme
in reaction with an RNA substrate is, to our knowledge, the
5   highest yet observed for any ribozyme with Mg^{2+} as the
activating ion. A value of k_{cat} of 41 min^{-1} has been reported
by Olsen et al. (Olsen, et al., 1991) for a hammerhead
ribozyme activated with Mg^{2+}, but the same ribozyme
activated by Mg^{2+} has a k_{cat} of only 2.1 min^{-1} at 25°C.
10

Experiments to measure the first-order rate constant for
cleavage of the substrate (data in Table 3) were conducted
under conditions where all the available substrate was
expected to be bound in ribozyme. The substrate
15   concentration of 500 mM was about 10-fold greater than the
k_m for the reactions, and the ribozyme was present in 3-fold
excess over the substrate. In addition, the ribozyme and
substrate were heated together in the absence of Mg^{2+}, and
then cooled together to the reaction temperature, thus
20   assisting the formation of ribozyme-substrate complexes.
The reaction was initiated by the addition of the Mg^{2+}
containing buffer. Under these conditions the rate-limiting
step is almost certainly the cleavage step, since the
ribozyme-substrate complex should be fully formed, the
25   addition of Mg^{2+} is expected to be rapid, and the product
dissociation is irrelevant in these conditions. Hence, the
observed rate constant is equal to k_2 in scheme 1 and should
equal k_{cat} for the turnover reaction, if in the turnover
reactions the cleavage step is rate-determining.
30

The rate constant, k_2, observed for the cleavage of S13 by
ribozyme 1, 5 min^{-1}, is slightly less than the kcat of 8.9
min^{-1} observed for the reaction under similar conditions.
This difference is not significant, given the following

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experimental limitations. In the turnover experiments to determine k_{cat}, absolute concentrations of both the ribozyme and the substrate are important in determining the kinetic parameters. Additionally, the trace amount of ribozyme used

5   make the results of the experiments subject to error due to absorption of the ribozyme on to the surface of the reaction vessels. In the experiments to independently determine k_2, the kinetic data are independent of absolute concentration of both ribozyme and substrate; however, in this case, the

10 rates of the reactions ($t_{1/2}$ ~ 8 seconds for ribozyme 2) make it difficult to determine the rate constants accurately. Therefore, it is reasonable to assume that the limiting step in the turnover reaction for ribozyme 2 is the cleavage. step.

15

On the other hand, the observed rate constant of 1.6 min^{-1} for the cleavage of S13 by ribozyme 1 is 4-fold greater than the k_{cat} of 0.4 min. for the turnover reaction. This discrepancy is too large to be accounted for by experimental

20 error, and it suggest that for ribozyme 1 the cleavage step is not rate-determining. In the cleavage of S13 under conditions where the ribozymes were in excess, less than 100% of the substrate was cleaved: this is due to the formation of inactive ribozyme-substrate complexes (McCall,

25 et al., 1992). Heat-pulsing the reaction mixture of 75°C for 3 minutes, followed by incubation of 30°C, allows the reaction to proceed further (data not shown). This observation suggests that the measured values of kcat underestimate the activity of the ribozymes in the active

30 complex. The error is likely to be more significant for ribozyme 1 than for ribozyme 2, since in the experiments with ribozyme in excess only 50% of S13 was cleaved by ribozyme 1 but 62% was cleaved by ribozyme 2 (Table 3). Assuming this to be the case for the turnover experiments as

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well, then the measured kcat of 0.4 min-1 for ribozyme 1
(the average for both the inactive and active complexes)
should be increased to ~ 0.8 min^{-1} for the active complex
alone. This value is closer to the independently-measured
k$_2$, but still differs from it by a factor of 2, and so
alternatives to the cleavage reaction being rate-determining
must be considered. If the dissociation of one or both
products from the ribozyme were rate limiting, a burst of
product would be observed at the beginning of the reaction;
this was not observed for either ribozyme. If the rate of
formation of complex were rate limiting for ribozyme 1, as
may be the case, then the likelihood is that ribozyme 1,
when uncomplexed, adopts a conformation which is not readily
able to bind the substrate. This conformation must be
stabilized, relative to ribozyme 2, by the all-RNA
hybridizing arms. The rate-limiting step then becomes the
rate of unfolding of this ribozyme into a form capable of
binding the substrate, and this unfolding rate may be
exacerbated by longer-than-necessary hybridizing arms.
Further experiments are necessary to unambiguously determine
the rate-limiting step for ribozyme 1.

The k$_m$'s for DNA and RNA substrates with all-RNA ribozymes
have been found to differ by 6- to 16-fold in one study
(Yang, et al., 1990) and were estimated to be 100-fold
greater for the DNA substrate in another (Dahm and
Uhlenbeck, 1990). In contrast, in our study where the DNA
is introduced into the ribozyme, the K$_m$ for ribozyme 2 with
the RNA substrate is only 1.5-fold greater than found for
ribozyme 1. In Michaelis kinetics, K$_m$ = (k$_{-1}$ + k$_2$)/k$_1$, k$_2$ has
been measured for both ribozymes and is 3-fold greater for
ribozyme 2, and k$_{-1}$ is expected to be greater for ribozyme 2
than ribozyme 1 because of the expected, relatively weaker
binding in DNA/RNA duplexes. It follows therefore that k$_1$

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for ribozyme 2 must also be greater than for ribozyme 1 in order for the difference in Km's to be as small as observed. This reduction in apparent k_1 for ribozyme 1 relative to ribozyme 2 supports the above speculation about an alternate conformation for ribozyme 1 which limits the rate of association with substrate.

Table 2. Results of turnover experiments with substrate S13

Ribozyme	k_{cat} (min^{-1})	K_m (nM)	k_{cat}/K_m (min$^{-1}\mu M^{-1}$)
ribozyme 1	0.40	38	10
ribozyme 2	8.9	59	150

Conditions: 30°C, pH 8.0, 10 mM MgCl$_2$

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Table 3. Rate constants and % product formed at infinite time (calculated) for the cleavage of substrate S13 (all-RNA) and substrate S21D (DNA with ribo C at cleavage site) by ribozymes 1 and 2 when in excess over substrate

Ribozyme	k_2 (min^{-1})	%P_∞
ribozyme 1 + S13	1.6 ± 0.6	49 ± 3
ribozyme 2 + S13	5.0 ± 1.0	61.5 ± 2
ribozyme 1 + S21D	0.044 ± 0.004	50 ± 1
ribozyme 2 + S21D	0.12 ± 0.01	70 ± 1

Conditions as for Table 2.

Cleavage of the DNA substrate

The cleavage of the DNA substrate, S21D, by the two ribozymes was performed under conditions where the ribozyme-substrate complex was expected to be fully formed prior to addition of Mg^{2+} to initiate the reaction. The substrate in this case was chosen to be 21 nucleotides to compensate for the expected weaker binding of ribozymes to a DNA substrate compared with an RNA substrate. Use of the longer substrate should not result in serious discrepancies in comparing data, since the rate of cleavage of the complex is not expected to depend strongly on the length of the substrate. Figure 6 shows an example of the data used to determine the rate constants. The data fit the first-order model well. The observed rate constants for cleavage of the DNA substrate are given in the lower half of Table 3. k_2 for ribozyme 2 (0.12 min^{-1}) is 3 times greater than for ribozyme 1, the same ratio as observed for cleavage of the all-RNA substrate by these ribozymes. The absolute values for rates of cleavage of the DNA substrate are approximately 40-fold less than observed of the DNA substrate are approximately 40-fold less than observed in earlier studies (Yang, et al., 1990; Dahm and Uhlenbeck, 1990). Why should DNA in the substrate decrease the rate of cleavage, but DNA in the arms of the ribozyme increase the rate? It is known that the 2'-

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hydroxyl of the uridine immediately 5' to the cleavage site
on the substrate is involved in binding the Mg^{2+} on (Yang,
et al., 1990). However, even if both that important uracil
and the cytosine at the cleavage site are ribonucleotides,
5   and the remainder are deoxyribonucleotides, such a substrate
is still not cleaved as efficiently as an all-RNA substrate
(Yang, et al., 1990). Therefore, either there are other 2'-
hydroxyl groups in the substrate that are directly involved
in stabilizing the active complex, or the structure of the
10   complex formed by the ribozyme and the predominantly DNA
substrate is sufficiently different from that formed by the
ribozyme with the all-RNA substrate to result in a slightly
different arrangement of the crucial groups involved in the
reaction. Our observation that activity does not decrease
15   when DNA is substituted into the arms of the ribozyme
indicates there are no crucial 2'-hydroxyl groups in the
arms, at least in positions 1-10 and 35-42 (Figure 5). This
results is consistent with the observations of Paolella et
al. (Paolella, et al., 1992) which showed complete retention
20   of activity by a ribozyme in which all 2'-hydroxyl groups in
the hybridizing arms had been replaced by 2'-O-allyl. The
fact that the rate of cleavage increases with DNA in the
arms of the ribozyme suggest that most likely there is a
subtle change in the conformation of the resulting double-
25   helix allowing a more favorable positioning of the critical
groups involved in the cleavage reaction. Thus, it may be
expected that differences between the effects of all-RNA
ribozymes and analogous DNA-armed ribozymes against specific
targets will vary with the sequence of the target, as the
30   local structure and flexibility of the helices formed will
depend also on the sequence.

Stability of ribozymes in serum
Ribozymes 1 and 2 were subjected to degradation in fetal

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calf serum in order to investigate their relative
stabilities. At all concentrations of serum between 5% and
0.01%, no significant differences were observed. At a serum
concentration of 0.1%, the half-life of both ribozymes was
5   around 1-2 minutes. Ribozyme 1 was cleaved into small
fragments with no preferred cleavage sites. Ribozyme 2, on
the other hand, was initially cleaved between G_{13} and A_{14} and,
subsequently, after T_{10} to yield a 10-mer of DNA which was
relatively stable and constituted >90% of the 5' end-
10   labelled material observed on the gel after 20 minutes
incubation of 0.1% serum. At higher serum concentrations
and longer times to 10-mer product was degraded to a 9-mer,
but no shorter fragments appeared even after 60 minutes in
5% serum. Clearly the DNA portion of ribozyme 2 is several
15   orders of magnitude more stable than the RNA. The
observation that both ribozyme 1 and ribozyme 2 are degraded
in serum at the same rate, without significant degradation
of the DNA portion of ribozyme 2, implies that RNA
endonucleases are predominantly responsible for the
20   degradation of this medium. In contrast, the DNA portion
appears to be very slowly degraded, largely, if not
exclusively, by 3' exonucleases.

CONCLUSIONS
25   The ribozymes described here differ in their efficiency, as
measured by k_{cat}/K_m, by a factor of 15. The directly-measured
first-order rate constants for the cleavage reactions differ
only 3-fold. The discrepancy probably arises largely from
different rate-determining steps for the two reactions.
30   Under the conditions used in this study, the rate-
determining step for the all-RNA ribozyme may be association
with substrate, whereas for the DNA-armed ribozyme it is the
cleavage reaction. The question of the rate-determining
step for ribozyme 1, while interesting, does not affect the

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conclusions that (i) a DNA-armed ribozyme is an order of
magnitude more active than its all-RNA analogue and (ii)
that this difference originates, in part, in the cleavage
step. If the observation holds generally, or even for a
5   subset of ribozymes, it means that DNA-armed ribozymes will
be very useful as starting molecules for the introduction of
further modifications designed to protect the conserved
nucleotides against nuclease attack: they possess enhanced
cleavage activity and nuclease resistant hybridizing arms.
10

Recently, Taylor et al. (Taylor, et al., 1992) have reported
data on a DNA-armed ribozyme that has 6-fold greater
catalytic activity than an analogous all-RNA ribozyme, when
targeted against a 28-mer RNA substrate at 55°C. They
15 attributed the difference in effect to the faster rate of
dissociation of the cleavage products from the DNA-armed
ribozyme. They also investigated the stability of the
ribozymes introduced into cells with Lipofectin, and found
that the DNA-armed ribozyme survived longer in cells than
20 the all-RNA ribozyme. Our observations, that a DNA-armed
ribozyme displays faster rates of cleavage and faster
turnover at 30°C compared to an all-RNA ribozyme, and the
observations of Taylor et al. (Taylor, et al., 1992), of
increased turnover rates on a different sequence, together
25 imply a general usefulness for these types of molecules.

Table 4 shows kinetic constants for DNA-armed ribozymes,
compared with all-RNA ribozymes and ribozymes consisting of
all DNA except for the conserved ribonucleotides. Data for
30 TATRA, TATRB, and TATRC may be found in Figure 7.

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Table 4

Ribozyme	k_{cat} (min^{-1})	K_m (nM)	$k_{cleavage}$ (min^{-1})	Temp (°C)
GHRA (all RNA)	0.4	38	1.6	30
GHRB (DNA arms)	8.9	59	5.0	30
TATRA (all RNA)	0.35	1084	0.24	37
TATRB (DNA arms)	1.8	1300	2.0	37
TATRC (DNA arms +DNA helix II)	0.45	1165	-	37

k_{cat} and K_m are from turnover kinetic experiments (ribozyme concentration very much less than substrate concentration); k_2 is the rate at which substrate is cleaved, and is measured with ribozyme concentration greater than substrate concentration. kcat will equal k_2 if the rate-determining step in the turnover reactions is the cleavage step.

All data were obtained using substrates of 13 nucleotides; 10 mM MgCl$_2$, 50 mM Tris,Cl pH 8.0. GH is growth hormone; substrate sequence 5' GCGGGUC AUGAAG 3' (SEQ ID NO:22). TAT substrate sequence 5' GGAAGUC AGCCUA 3' (SEQ ID NO:23).

The important and unexpected observation here is that the rate of cleavage of the substrate by ribozyme is increased several-fold when DNA is in the hybridizing arms of the ribozyme, compared to an all-RNA ribozyme. (Note that we expected better stability properties when we introduced DNA into the minizyme, and that is why we continued on with the DNA-armed ribozyme experiments; however the enhanced cleavage activity we obtained with DNA-armed ribozymes was certainly not an expected result).

Figure 8 shows that activity of minizymes and ribozymes against long and short substrates. Suprisingly, the minizyme is much more active against the long substrate.

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Experiment 3

Number of nucleotides in hybridizing arms or ribozyme.

(a) Four DNA-armed ribozymes were synthesized which were
5   identical except for the number and sequence of the
nucleotides in the hybridizing arms. In the molecules
below, lower case letter represent DNA, upper case letters
RNA, x + y shows the number of potential base pairs formed
between the ribozyme and substrate, and the nucleotides
10   involved in these potential base-pairs are underlined.

GHRB 5'<u>gacacttcat</u>CUGAUGAGUCCUUUUGGACGAAA<u>Cccgcaggt</u> 10 + 10
+ substrate 5' ACCUGCGGGUC AUGAAGUGUC (SEQ ID NO:24-25)

15   TATRB 5'<u>gtcctaggct</u>CUGAUGAGUCCUUUUGGACGAAA<u>Cttcctgga</u> 10 + 10
+ substrate 5' UUCCAGGAAGUC AGCCUAGGAC(SEQ ID NO:26-27)

TNFRB 5'<u>aagatgatct</u>CUGAUGAGUCCUUUUGGACGAAA<u>Ctgcctgg</u> 10 + 9
+ substrate 5' CCAGGCAGUC AGAUCAUCUU (SEQ ID NO:28-29)
20

ILKRB   5'<u>caatgcaa</u>CUGAUGAGUCCUUUUGGACGAAA<u>Cagga</u>   8 + 6
+ substrate 5' UCCUGUC UUGCAUUG(SEQ ID NO:30-31)

The cleavage activities of these ribozymes, against their
25   respective short substrates, are given in Figure 9. Ribozyme
is in excess over substrate; 10 mM MgCl$_2$, 50 mM Tris.Cl, pH
8, 37°C.

(b) In addition to the cleavage activities by GHRB and TATRB
30   against 21-nucleotide substrates shown in (a) above, we have
measured cleavage activities by GHRB and TATRB against
shorter, 13-nucleotide substrates. The substrate sequences
(SEQ ID No.:32-33) are

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GH   5' GCGGGUC AUGAAG
TAT   5' GGAAGUC AGCCUA.

and so these provide two examples of 6 + 6 base pairs formed
5   between DNA-armed ribozyme and RNA substrate.

The cleavage activities against the shorter substrates are
shown in Figure 10, with data for the analogous all-RNA
ribozymes (GHRA and TATRA) also included. For reasons we do
10   not yet understand, both GHRB and TATRB cleave their
respective 13-mer substrates faster than their 21-mer
substrates.

3.   Number of base pairs in stem of helix II.
15

In addition to the minizyme which has zero b.p. in helix II,
we have DNA-armed ribozymes which are identical in every way
except for the number of base pairs in helix II; and we have
examples of 2, 4 or 8 b.p. in helix II. In the molecules
20   below, the nucleotides involved in base-pairs in helix II
are underlined. TATRB-H4 (below) is identical to TATRB
(above)(SEQ ID NO:34-35). (See Figure 11)

TATRB-H2
25   5'gtcctaggctCUGAUGAG<u>C</u>UUUUG<u>C</u>GAAACttcctgga

TATRB-H4
5' gtcctaggctCUGAUGA<u>GUCC</u>UUUU<u>GGAC</u>GAAACttcctgga

30   TATRB-H8
5' gtcctaggctCUGAUGA<u>GUCCGUCC</u>UUUU<u>GGACGGAC</u>GAAACttcctgga

The cleavage activities of these ribozymes against the same
short substrate is shown in Figure 12, and that against a
35   longer transcribed substrate which contains the target

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sequence is shown in Figure 13.

4.   DNA-armed ribozymes with modified nucleotides are shown in Figure 14.

TATRBPS is similar to TATRB above, except that phosphates are replaced by phosphorothioates in the stem-loop of helix II (shown by capital letter in italics).

The activity of this ribozyme, relative to TATRG and TATRH, is shown in Figure 15.

5.   Increased stability in cells.
There is data that DNA-armed ribozymes are much more stable in cells than all RNA ribozymes (Taylor, et al., 1992).

Experiment 4
As part of a program to develop minizymes as pharmaceuticals, we are investigating whether non-nucleotide moieties may replace helix II.   In this study, we have prepared three series of minizymes in which helix II is replaced by linkers containing various numbers of neutral ethyleneglycol units, charged phosphopropanediol units, or charged deoxyribothymidine units.   A comparison of the cleavage activities of these minizymes against the same substrate allows the optimal number of atoms in the chain linking the two stretches of conserved nucleotides to be determined, and gives some insight into the chemical properties required by the linker.

MATERIALS AND METHODS

Synthesis   of   the   phosphoramidite   of   the   mono-dimethoxytrityl-protected   hexaethyleneglycol   was   as described by Durand et al (Durand, 1990) and that of the

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tetra-ethyleneglycol phosphoramidite was analogous.

Synthesis of the phosphoramidite of mono-dimethoxytrityl-protected 1,3-propanediol was by a modification of the
5   procedure of Seela and Kaiser (Seela, 1987).   1,3-propanediol and dimethoxytrityl chloride were purchased from
Aldrich   Chemical   Co.   (Milwaukee,   WI,   USA).   The
phosphitylating   reagent,   2-cyanoethyl-N,N-diisopropylchlorophosphoramidite   was   purchased   from
10   ChemGenes Corp. (Waltham, MA, USA). Diisopropylethylamine
was distilled over potassium hydroxide, and tetrahydrofuran
(THF) was dried with sodium under nitrogen. Dichloromethane
(DCM) was dried over 4-A molecular sieves. Analytical thin
layer chromatography (TLC) was performed on silica gel 60
15   F_{254} plates (Merck). The eluant systems for TLC were: system
A   (DCM),   system   B   [DCM/MeOH   (200/1,   v/v)],   system   C
[Petroleum   ether/ethyl   acetate/   triethylamine   (20/10/1,
v/v/v)].   Nuclear   magnetic   resonance   (NMR)   spectra   were
recorded with a 200 MHz Bruker spectrophotometer.
20

1-O-(4,4'-dimethoxytrityl)-3-propanediol

1,3-Propanediol (2.28 g 30 mmol) was dissolved in 60 ml of
dry DCM, and diisopropylethylamine (DIPEA) (2.6 g, 20 mmol)
25   was added to the solution. Dimethoxytrityl chloride (DMT-Cl) (2.03 g, 6 mmol), dissolved in 50 ml dry DCM, was
dropped over 90 min at room temperature under nitrogen
atmosphere. Progress of the reaction was followed by TLC,
System A and B. After 3 hours the solution was washed with
30   5% aqueous sodium bicarbonate (50 ml) and water, and the
organic phase was dried over anhydrous sodium sulphate,
filtered and evaporated. The residual oil was purified by
flash chromatography on silica gel. The column was first
washed with DCM and then with DCM/MeOH/triethylamine

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(98/1/1, v/v/v). Fractions containing the monotritylated
1,3-propanediol were combined and the solvent was evaporated
and dried overnight under high vacuum. The yield, based on
the starting DMT-Cl, was 86%. TLC (Silica gel, System A),
5   Rf: 0.17, (System B), Rf: 0.47, ^{1}H-NMR (CDCl$_3$): d 1.85 (2H,
quint, CH$_2$CH$_2$CH$_2$); 2.26 (1H, s, OH); 3.26(2H, t, DMT OCH$_2$);
3.74-3.80 (2H, m, CH$_2$CH$_2$OH); 3.80 (6H, s, OCH$_3$); 6.82 (4H, m,
arom. H ortho of OCH$_3$); 7.15-7.45 (9H, m, arom. H).

10   *1-O-(4,4'-dimethoxy trityl)-3-O-[(N,N-diisopropyl amino) ß-*
     cyanoethoxy phosphino]-1,3-propanediol

The monotritylated compound (0.378 g, 1 mmol) was dissolved
in 3 ml dry THF and preflushed with nitrogen. Anhydrous
15   DIPEA (0.68 ml, 4 mmol) was added to the stirred solution by
syringe. The phosphitylating reagent, 2-cyanoethyl-N,N-
diisopropylchlorophosphoramidite (0.47, 2 mmol), was added
by a syringe over a period of 2-3 min under a weak flow of
nitrogen. The reaction mixture was stirred at room
20   temperature, and the reaction progress was followed by TLC
(system C). After 30 min, several ice cubes were added to
the reaction mixture to quench the excess phosphitylating
reagent. Ethyl acetate (10 ml) and triethylamine (1 ml)
were added to the mixture, which was then washed with 10 ml
25   aqueous sodium bicarbonate and water. The organic phase was
dried over anhydrous sodium sulphate, filtered and
evaporated. The residue was purified by flash
chromatography on silica gel using the system C eluant.
Fractions containing pure product were combined, evaporated
30   and dried overnight under high vacuum, yielding a colorless
oil (0.34 g, 60%). TLC (silica gel, system C), R_f: 0.78, ^{1}H-
NMR (CDCl$_3$: d 1.1-1.3 [12 H, 2d, CH(CH$_3$)$_2$; 1.9-2.0 (2H, m,
CH$_2$CH$_2$CH$_2$); 2.45-2.55(2H, t, CH$_2$ CN), 3.1-3.2 (2H, t,
DMTOCH$_2$); 3.5-3.9 (12H, m, POCH$_2$CH$_2$CN, CH$_2$OP, OCH$_3$); 6.75-6.88

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(4H, m, arom, *H* ortho of OCH_3); 7.15-7.50 (9H, m, arom. *H*).

Oligonucleotides were synthesized using an Applied Biosystems model 391 DNA synthesizer. Protected DNA
5 phosphoramidite monomers were from Applied Biosystems. Protected RNA monomers, protected at the 2'-hydroxyl with tertbutyldimethylsilyl groups, were from Milligen. For convenience in the syntheses, almost all of the oligonucleotides have a deoxyribonucleotide at their 3' end
10 as shown in Figure 16. Deprotection and purification of oligonucleotides were as described previously (Hendry et al., 1992). The purity of each oligonucleotide was checked by labelling its 5'-end with ^{32}P phosphate using T4 polynucleotide kinase (new England Biolabs, Beverly MA USA)
15 and γ-^{32}P ATP (Bresatec, Adelaide, S.A. Australia), electrophoresing the molecules on a 10-15% polyacrylamide gel containing 7M urea, and visualizing the molecules by autoradiography or using a Molecular Dynamics PhosphorImaging system; all oligonucleotides were at least
20 98% pure, as judged by this assay. The concentrations of the purified oligonucleotides were determined by UV spectroscopy using the following molar extinction coefficients for the various nucleotides at 260 nm: A, 15.4 x 10^3; G, 11.7 x 10^3; C, 7.3 x 10^3; T/U, 8.8 x 10^3 liter mol$^-$
25 ^{1}cm^{-1}. All oligonucleotides were stored in either water or 10 mM Tris.Cl, pH 8.0, 0.2 mM EDTA at -20°C.

Kinetic experiments with minizymes and the ^{32}P-labelled substrate S13 (Table 5) were conducted in 50 mM Tris.Cl, pH
30 8, 10 mM $MgCl_2$, at 30°C, with the concentration of minizyme at least 1.5-fold greater than that of substrate. Typically, the substrate concentration was in the region of 1 to 2 μM, and the minizyme 2 to 5 μM. The minizyme and substrate were heated together in the Tris buffer at 80°C

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for 2 minutes, then allowed to cool to 30°C for 2-3 minutes, before initiating the reaction by adding 1/10 volume of 100 mM MgCl$_2$. 2μL samples were removed at appropriate time-intervals and added to 4μL of 90% formamide, 20 mM EDTA,

5   0.1% bromophenol blue, and 0.1% xylene cyanol, to quench the reaction. The extent of cleavage at each time point was determined by electrophoresing the samples in a 15% polyacrylamide gel containing 7 M urea and Tris-Borate-EDTA buffer, and quantifying the relative intensities of bands

10   corresponding to the 5' cleavage product and the uncleaved substrate using a Molecular Dynamics PhosphorImager and ImageQuant software. The kinetic parameters were obtained by fitting the data for percentage of product formed *versus* time to the equation:

15         $P_t = P_\infty - (\exp(-k_2 t) P_D)$

where P_t is the percentage of product at any given time, P_∞ is the percentage of product at t=∞, k_2 is the first-order rate constant for the reaction, t is the time, and P_D is the difference between the percentage of product at t=∞ and t=0.

20   This is a conventional first-order kinetic equation from which k_2, P_∞ and P_D are determined by least-squares fitting of the data. The quoted rate constants and P_∞ values in Table 5 are the mean ($\pm$SD) for at least 2 independent experiments.

25

RESULTS

Figure 16 shows the sequences of the molecules used in this study. Near the top of Figure 16A are the sequences of two

30   "half-minizymes," KrM½A and KrM½B. KrM½A contains the conserved nucleotides CUGA(U)GA at its 3'-end, while KrM½B contains the conserved nucleotides GAAA at its 5'-end. When the two half-minizymes are mixed in solution with their respective substrate, KrS21, at 25°C, they cleave

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approximately 5% of the substrate in three hours (data not
shown). The site of cleavage is 3' to the cytidine in the
triplet GUC, near the middle of KrS21. Thus these two half-
minizymes, which have no atoms linking the conserved 3'A9
5   (of KrM½A) to the conserved 5'G12 (of KrM½B), are together
able to cleave their substrate at the same place as does a
hammerhead ribozyme.   This suggests that the active
conformation of a hammerhead ribozyme is able to be formed
by the conserved nucleotides alone; however, in the absence
10   of helix II or any other atoms connecting the two stretches
of conserved nucleotides, the active structure is very
unstable, perhaps just forming transiently, and is able
therefore to cleave the substrate only very slowly.

15   These results indicate that a role for helix II is to
stabilize the active structure of the ribozyme, either by
holding the two free ends of the conserved nucleotides so
that they are an appropriate distance apart from each other
in space, and/or by contributing to the stability of the
20   active   structure   through   some   form   of   base-stacking
interactions.   Therefore we decided to make a ribozyme in
which helix II was replaced by a flexible chain which linked
the two stretches of conserved nucleotides, but which
contained no bases, to see if simply tethering the conserved
25   regions would improve the activity of the ribozyme.

A linker consisting of repeated units of phosphopropanediol
moieties was chosen first, because each phosphopropanediol
monomer has the same number and types of atoms in its chain
30   as in the sugar-phosphate chain of a nucleotide within a
strand of nucleic acid.   The sequences of five minizymes
synthesized with phosphopropanediol linkers of increasing
length are shown as M(n)pd in Figure 16, where pd represents
a monomer of phosphopropanediol as shown in Figure 17, and
35   n represents the number of phosphopropanediol moieties in

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each linker. The minizymes were reacted with a 13-
nucleotide substrate, S13 (Figure 16), in 10 mM MgCl$_2$, 50 mM
Tris. HCl, pH 8, at 30°C, as described in Materials and
Methods. The rate constants for cleavage of the substrate
5   S13 by these minizymes M1pd, M2pd, M3pd, M4pd and M5pd, and
the number of atoms in the chain between the 3' and 5'
oxygens of conserved nucleotides adjacent to the linker, are
shown in Table 5. A graph showing the relation between rate
constant and number of atoms in the linker is shown in
10   Figure 18. There is no discernable cleavage of substrate in
2 hours by M1pd which has only seven atoms in its linker.
Thereafter, rate constants increase with increasing linker
length, until a plateau apparently is reached with four
phosphopropanediol moieties (25 atoms) in the linker.
15

Two minizymes containing a second type of non-nucleotide
linker, repeated units of ethyleneglycol, were also
synthesized. Their sequences are shown as Meg in Figure 16,
and the full structures of the two linkers containing
20   tetraethyleneglycol (in Mteg) and hexaethyleneglycol (in
Mheg) are drawn in Figure 17. The minizymes were reacted
with substrate S13, and the rate constants for cleavage and
the number of atoms in the linkers are shown in Table 5.
Rate constants as a function of linker length are also
25   plotted in Figure 18. These minizymes follow the trend of
increasing activity with increasing linker length, as
observed with the minizymes containing phosphopropanediol
linkers. However, both Mteg and Mheg are slightly less
active than would be expected from a simple inspection of
30   linker length, when compared with the phosphopropanediol
series (see Figure 18).

A series of five minizymes containing linkers with repeated
units of thymidine residues has been made; their sequences

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are shown as M(n)t in Figure 16, where t represents a
thymidine monomer, and n the number of monomers in each
linker. Their rate constants for cleavage of the substrate
S13 and the number of atoms in the linker are shown in Table

5   5; and data for rate constants as a function of linker
length are added to Figure 18. In this series of minizymes,
the rates of cleavage of the substrate increase, with
increasing numbers of thymine deoxyribonucleotides in the
linker, to a maximum value when n=5 (31 atoms); the rate

10   slightly decreases on the addition of a further thymidine.
The rate constant for M5t is 230-fold greater than for M2t.
The variation in rate constants observed for the series of
minizymes with thymidine linkers is much greater than that
observed for the series with phosphopropanediol linkers, but

15   the actual rates of both types start to plateau with linkers
of 25 atoms (Figure 18).

The cleavage rates for the minizymes M(n)t (with n=2-5), in
reactions with a 21-nucleotide substrate at 37°C, have been

20   reported previously (McCall et al., 1992). The cleavage
rates presented in Table 5 for these same minizymes M(n)t
are for reactions with the 13-nucleotide substrate S13 at
30°C. In the present study, we chose to use the shorter
substrate in order to minimize possible dimer or hairpin

25   formation by the substrate which might complicate the
analysis of the kinetic data. Since the minizymes M(n)t
were originally made for reactions with the 21-nucleotide
substrate, they all have 10 nucleotides in each hybridizing
arm, and hence they differ from the other minizymes used in

30   this study which have only 6 nucleotides in each hybridizing
arm. In order to check whether the length of the
hybridizing arms influences cleavage rates of the minizymes,
we synthesized M4t(6x2), which has six nucleotides in each
of its hybridizing arms and four thymidines in its linker

35   (Figure 16), and compared its rate of cleavage of the 13-mer

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substrate with that of the minizyme M4t. The rate constant of 5.9 x 10^{-2} min^{-1} for M4t(6x2) was slightly lower than that of 7.4 x 10^{-2} min^{-1} for M4t (Table 5). However, the difference between these two rate constants is much smaller than the difference between rate constants from M4t(6x2) and M4pd (the two minizymes which have the same number of atoms in the linker and the same number of nucleotides in the hybridizing arms), and so a comparison of the rate constants for the series of minizymes M(n)t with those for the series M(n)pd and Meg should allow valid conclusions to be drawn.

Thymidine residues were chosen for the nucleotide-containing linkers, because we thought these would produce the most flexible linkers of this type. Since the thymidine-containing linkers are probably more structured than the very flexible non-nucleotide linkers studied, and they are better at activating a minizyme when four or more thymidine units are used, we wondered whether an even more structured nucleotide linker would better activate a minizyme. The nucleotides UUCG form a very stable loop in double-helical RNA (Antao and Tinoco, 1992), and so we used this sequence to make the minizyme M$_{UUCG}$ (Figure 16). The minizyme with UUCG in its linker is 60-fold less active than the analogous minizyme M4t with TTTT in its linker (Table 5).

For purposes of comparison, the rate constant for cleavage of the substrate S13 by the hammerhead ribozyme with DNA in the flanking sequences (11) (GHRB in Figure 16) is 5 min^{-1}.

DISCUSSION

The relative activities of all minizymes used in this study have been assessed by comparing rate constants for the cleavage step, k_2, as determined from experiments with minizyme in excess of substrate, rather than Michaelis-

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Menton constants, k_{cat} and K_m, determined from turnover experiments with substrate in excess of minizyme. We chose to measure cleavage rate constants directly for several reasons.   Firstly, by using the small 13-nucleotide

5   substrate, the rate constant for the cleavage reaction is likely to be slow compared with the rates of association of the substrate with the minizymes, so that the observed rate constants reflect the cleavage step, which is the critical step in a study of this type.   Secondly, rate constants

10   determined with minizyme in excess are not critically dependent on accurate measurements of minizyme and substrate concentrations, unlike k_{cat} and K_m, and so a comparison of k_2 values should be more reliable.

15   All three types of linker studied here show a common trend of increased rates of cleavage with increasing length (Table 5 and Figure 18), with maximal rates being reached, in the series of minizymes M(n)pd and M(n)t, with linkers of about 25 atoms.   The lack of reaction observed for M1pd, and the

20   slow reactions observed for M2pd and M2t, indicate that the linker must contain a minimum number of atoms, between 8 and 13, in order for the minizyme to show activity. Presumably, shorter linkers do not allow the terminal A9 and G12 nucleotides (of the two strings of conserved nucleotides

25   CUGAUGA and GAAA, respectively) to achieve the spatial separation required for them to readily adopt the active conformation for cleavage.   Increasing linker length relieves this strain, so that rates of cleavage increase, until an optimal linker length is reached where no strain is

30   placed on the positioning of A9 and G12 (Numbering as in Hertel et al., 1992).   Thus, tethering the two strings of conserved nucleotides results in improved activity, as compared to no connection, provided the tether is long enough.

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The activities of the minizymes with ethyleneglycol linkers are 2-fold lower than would be expected when compared with the minizymes with phosphopropanediol linkers, if the comparison is based on linker length alone (Figure 18).

5   Thus, while simply tethering the conserved A9 and G12 nucleotides results in improved activity, the type of chemical tether used adds an additional component to the magnitude of the activity observed. The most obvious chemical difference between the phosphopropanediol and

10   ethylenglycol linkers is the presence in the phosphopropanediol chains of regularly-spaced negative charges residing on the phosphates. This could influence the activity of the minizymes in several ways. One possibility is that the regular positioning of negative

15   charges in the spacer molecule will electrostatically "stretch" the linker, which would otherwise be shorter because of entropic effects. Another possibility is that the active conformation adopted by the conserved nucleotides has a preference for negatively-charged species in this

20   region.

For chains of identical length in the two series of minizymes with phosphopropanediol and thymidine linkers, the relative rates of cleavage depend strongly on the actual

25   number of atoms in the connecting chains (Figure 18). Thus, with linkers of 13 and 19 atoms, the phosphopropanediol-containing minizymes are the more active, while for longer linkers, the minizymes with thymidine linkers are more active. Both types of minizymes show maximal activity at

30   the same chain-length of 25 atoms. Thus, there appears to be at least two interplaying factors which determine activity. The presence of the deoxyribose ring and the thymine base in very short linkers probably tends to interfere with the ability of the chain to span the required

35   distance between the conserved A9 and G12 nucleotides; but

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as the linker gets longer, the negative steric influences of the nucleosides become less important and a positive effect, possibly the stacking of one or more of the thymine bases with bases in the conserved domain, adds stability to the structure.

The distance to be spanned between the 3'O of A9 and the 5'O of G12 of a hammerhead ribozyme appears to be met by 25 atoms in a flexible chain. In the light of this result, the observation that the loop-forming sequence UUCG, when placed in the linker of a minizyme, results in a 60-fold reduction in activity compared with the more flexible sequence TTTT, can now be interpreted in terms of effective linker length. Although the linker UUCG contains 25 atoms, the stable structure of this loop prevents it from stretching the distance achieved by the more flexible chains with this atom count, and so M_{UUCG} has the activity of a minizyme with an apparently shorter linker. As discussed below, the minimum number of atoms in a flexible linker that spans a normal double-helix is of the order of 13, and so it is interesting (although possible coincidental) that the rate constant for M_{UUCG} is similar to those for M2pd (13 atoms in the linker) and Mteg (15 atoms).

Relation to other work.
Using various types of spacer molecules, several studies have determined the number of atoms required to span the ends of the two polynucleotide-strands of a normal double-helix. Durand et al. (Durand, 1992) found that a hexaethyleneglycol linker of 21 atoms enable both duplex and triplex formation by the molecule $(dA)_{12}$-heg-$(dT)_{12}$-heg-$(dT)_{12}$. Ma et al. (Ma, 1993) used molecular-modelling programs and α,"-alkanediol spacers between terminal phosphate groups to calculate that a minimum of 9 methylene-carbon atoms were

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required to effectively span the ends of a double helix of
RNA; this corresponds to a spacer of 13 atoms between the
terminal 5' and 3' oxygen atoms. In experimental tests (Ma,
1993), the natural 6-nucleotide loop of the TAR sequence of

5   HIV was replaced with a spacer of 13 atoms, based on 1,9-
nonanediol, and this modified TAR molecule retained the
melting temperature and TAT-peptide binding-ability of the
wild-type molecule, while another modified TAR molecule
containing a shorter linker of 7 atoms had reduced T_m and

10   did not bind the TAT peptide. Baxter et al. (Baxter, 1993)
found that DNA double-helices with loops of either three or
four deoxyribothymidines had the same melting temperatures,
and their NMR data indicated that these loops had very
flexible structures; in contrast, our minizyme M3t, with

15   three deoxyribothymidines in the linker, is an order of
magnitude less active than M4t. It appears, therefore, that
the distance between terminal atoms of the strands of normal
double-helix of DNA or RNA is considerably less than the
distance between the terminal, conserved A9 and G12

20   nucleotides in a minizyme or ribozyme.

Benseler et al. (Benseler, 1993) used a variety of linkers
to replace the loop at the base of helix II in a hammerhead
ribozyme, and progressively reduced the number of base pairs

25   in helix II to produce some minizymes with non-nucleotide
linkers similar to those we have studied. The cleavage
rates for their minizymes which contained the same linkers
as used in this study, 5.2×10^{-3} min^{-1} (hexaethyleneglycol),
6×10^{-3} min^{-1} (2X propanediol), and no cleavage $(3 \times$

30   propanediol) were in good agreement with those measured by
us, with the exception that the relative activities of the
last two minizymes appear to be reversed. We have no
explanation for this discrepancy, but the lower activity for
M2pd, rather than for M3pd, is consistent with the increase

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in activity observed with increasing linker length for all
the minizymes in this study. In addition, the data of
Benseler et al. (Benseler, 1993) show that the number of
base pairs in helix II can be reduced to two, with full
5 retention of cleavage activity, if the non-nucleotide linker
is the neutral hexaethyleneglycol, but not if the linker
contains charged phosphodiester groups.   The situation
appears to be different in the minizyme where the linker
abuts the conserved domain, since our data for the series of
10 minizymes containing ethyleneglycol and phosphopropanediol
linkers show that the presence of a negatively charged
phosphate groups appears to enhance cleavage rates for a
given chain length.

15 In summary, we have shown that (i) a string of atoms linking
the conserved nucleotides A9 and G12 in a hammerhead
ribozyme enhances activity, as compared to no connection,
provided the string is sufficiently long, (ii) the linker
must contain a minimum of 25 atoms for the minizyme to have
20 optimal activity, (iii) the linker may consist of non-
nucleotide moieties.

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Table 5.

Rate constants for cleavage of S13 by various minizymes at 30°C.

minizyme	k_2 (min^{-1})	%P_∞	linker length (atoms)
M1pd	No reaction observed		7
M2pd	$(1.80\pm0.06) \times 10^{-3}$	90*	13
M3pd	$(6.0\pm0.2) \times 10^{-2}$	92±2	19
M4pd	$(1.7\pm0.1) \times 10^{-2}$	92±1	25
M5pd	$(1.8\pm0.1) \times 10^{-2}$	89±1	31
Mteg	$(1.6\pm0.2) \times 10^{-3}$	90*	15
Mheg	$(3.9\pm0.3) \times 10_{-3}$	95±1	21
M2t	$(4\pm1) \times 10^{-4}$	90*	13
M3t	$(4.7\pm0.1) \times 10^{-3}$	78±1	19
M4t	$(7.4\pm0.2) \times 10^{-2}$	94±1	25
M5t	$(9.7\pm0.7) \times 10^{-2}$	90±2	31
M6t	$(6.5\pm0.1) \times 10^{-2}$	88±3	37
M4t(6x2)	$(5.9\pm0.7) \times 10^{-2}$	92±2	25
MUUCG	$(1.2) \times 10^{-3}$	90*	25
GHRB	5±1	63±2	—

*   In these cases, the reaction rate was too slow to follow for a sufficient period of time to allow an accurate determination of %P_∞ and so, for the purposes of calculating k_2, it was fixed at 90%, which is in the range observed for all the minizyme reactions.

Experiment 5

The rigid linker in Figure 19 was incorporated in to the minizymes utilizing the standard phosphoramidite chemistry;

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the purification and workup of the minizymes was as normal.
We have incorporated the carbazole moiety into two new
minizymes: one with the carbazole replacing the linker
region (GH M Carb) and the other with the carbazole flanked

5   by phosphopropanediol moieties as the linker region (GH M
pcp). The carbazole linkers are incorporated into the same
minizyme as described in the flexible linker paper. The
kinetics were performed by the same method and using the
same 13-mer RNA substrate as described in experiment 4.

10

The constants for cleavage of substrate at 30°C, 10mM $MgCl_2$,
50 mM Tris.Cl pH 8.0 are as follows:

GH M Carb: -2×10^{-4} min^{-1}

15   GH M pcp: $(1.0 \pm 0.1) \times 10^{-2}$ min^{-1}

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What is claimed is:

1.   A compound having the structure:

```
3'  (X)ₙ——A              X——(X)ₙ·   5'
           A              C
           A               \U
           |                  \  G
           G                    \A
            \         A—G— N´
             \ (X)ₘ /
```

wherein each X is the same or different and represents a ribonucleotide or a deoxyribonucleotide which may be modified or substituted in its sugar, phosphate or base;

wherein $(X)_n$ and $(X)_{n'}$ represent oligonucleotides in which n and n' are integers which define the number of nucleotides in the oligonucleotides, such oligonucleotides having predetermined sequences sufficiently complementary to a predefined RNA target sequence to be cleaved to allow hybridization to the RNA target sequence;

wherein N may be adenine, guanine, cytosine or uracil;

wherein each solid line represents a chemical linkage providing covalent bonds between the nucleotides located on either side thereof;

wherein m represents an integer from 2 to 20; and wherein none of the nucleotides $(X)_m$ are Watson-Crick base paired to any other nucleotide within the compound.

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2.   The compound of claim 1 having the structure:

$$3' \quad (X)_{n-1}-CA \qquad\qquad X-(X)_{n'} \quad 5'$$

```
3'  (X)ₙ₋₁—CA              X——(X)ₙ'   5'
              A                 C
              A                  ⟍U
              |                     ⟍G
                                      ⟍A
              G                A—G——N⟍
               ⟍(X)ₘ⟋
```

wherein each X is the same or different and represents a ribonucleotide or a deoxyribonucleotide which may be modified or substituted in its sugar, phosphate or base;

wherein $(X)_n$ and $(X)_{n'}$ represent oligonucleotides in which n and n' are integers which define the number of nucleotides in the oligonucleotides, such oligonucleotides having predetermined sequences sufficiently complementary to a predefined RNA target sequence to be cleaved to allow hybridization to the RNA target sequence;
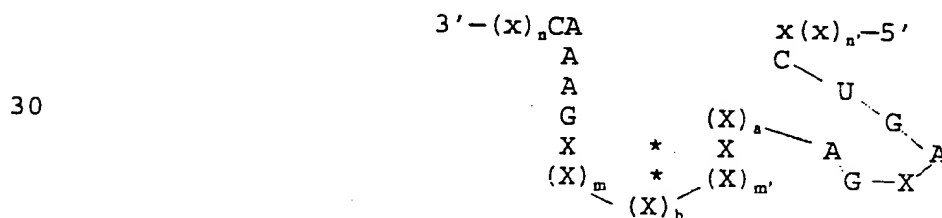
wherein N may be adenine, guanine, cytosine or uracil;

wherein each solid line represents a chemical linkage providing covalent bonds between the nucleotides located on either side thereof;

wherein m represents an integer from 2 to 20; and wherein none of the nucleotides $(X)_m$ are Watson-Crick base paired to any other nucleotide within the compound.

3.   A compound of claim 1, wherein each of X represents a ribonucleotide.

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4.   A compound of claim 1, wherein each X represents a
     deoxyribonucleotide.

5.   A compound of claim 1, wherein m is an integer from 2
     to 6.

6.   A compound having the structure:

              3'   [-(Y)$_r$——Q——(Y)$_s$-]$_z$ 5'

     wherein Q represents a compound of claim 1 which may be
     the same or different;

     wherein each Y represents a ribonucleotide or a
     deoxyribonucleotide which is the same or different and
     may be modified or substituted in its sugar, phosphate
     or base;

     wherein each of r and s represents an integer which is
     greater than or equal to 0; and

     wherein z represents an integer from 1 to 100.

7.   A pharmaceutical composition comprising an amount of
     the compound of claim 1 in association with a
     pharmaceutically acceptable carrier or excipient.

8.   A method for producing the compound of claim 3, which
     comprises the steps of:

          (a)   ligating into a transfer vector comprised of
                DNA, RNA or a combination thereof a
                nucleotide sequence corresponding to said
                compound;
          (b)   transcribing the nucleotide sequence of step

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(a) with RNA polymerase; and

(c)   recovering the compound.

9.   A transfer vector comprised of RNA or DNA or a
     combination thereof containing a nucleotide sequence
     which on transcription gives rise to the compound of
     claim 3.

10. A transfer vector according to claim 9 which is a
     bacterial plasmid or phage DNA.

11. A prokaryotic or eukaryotic cell containing a
     nucleotide sequence which on transcription gives rise
     to the compound of claim 3.

12. A method of cleavage of a specific RNA target sequence
     which comprises reacting the compound of claim 1 with
     the target sequence so as to thereby cleave the
     specific target sequence.

13. The method of claim 12, wherein the target sequence is
     indigenous to a mammal.

14. The method of claim 12, wherein the target sequence is
     indigenous to a plant.

15. The method of claim 12, wherein the target sequence is
     in a viral gene.

16. A method for the treatment of a viral disease in
     mammals which comprises administering to a mammal an
     effective amount of the compound of claim 1 capable of
     cleaving the viral gene so as to thereby cleave the
     viral gene and treat the viral disease.

17. A method according to claim 16, wherein the virus is a
     human virus such as papillomavirus, hepatitis virus,
     Epstein-Barr virus, human T cell leukemia virus, HIV,
     herpes virus, cytomegolovirus, influenza virus,
5   varicella-zoster virus, respiratory syncytial virus,
     rhinovirus, or picornovirus.

18. A compound having the structure:

$$3'-(x)_n\ A \qquad\qquad x(x)_{n'}-5'$$
$$A \qquad\qquad C$$
$$A \qquad\qquad\qquad U$$
$$G \qquad (X)_a \qquad\qquad G$$
$$X \quad * \quad X \qquad A \qquad A$$
$$(X)_m \quad * \quad (X)_{m'} \qquad G-X$$
$$(X)_b$$

     wherein each X represents a ribonucleotide which
     is the same or different and may be modified or
     substituted in its sugar, phosphate or base;

     wherein each x represents a deoxyribonucleotide
     which is the same or different and may be
     modified or substituted in its sugar, phosphate
     or base;

     wherein each of A, C, U, and G represents a
     ribonucleotide which may be unmodified or
     modified or substituted in its sugar, phosphate
     or base;

     wherein each of $(x)_n$ and $(x)_{n'}$ represents an
     oligodeoxyribonucleotide having a predetermined
     sequence and each of n and n' represents an
     integer which defines the number of
     deoxyribonucleotides in the oligonucleotide with

the proviso that the sum of n + n' is sufficient to allow the compound to hybridize with the RNA target sequence;

wherein each * represents base pairing between the nucleotides located on either side thereof;

wherein each solid line represents a chemical linkage providing covalent bonds between the ribonucleotides located on either side thereof;

wherein a represents an integer which defines a number of ribonucleotides with the proviso that a may be 0 or 1 and if 0, the A located 5' of $(X)_a$ is bonded to the X located 3' of $(X)_a$;

wherein each of m and m' represents an integer which is greater than or equal to 1;

wherein $(X)_b$ represents an oligoribonucleotide and b represents an integer which is greater than or equal to 2.

19. The compound of claim 18 having the structure:

$$3'-(x)_nCA \qquad\qquad x(x)_n-5'$$
$$A \qquad\qquad\qquad C$$
$$A \qquad\qquad\qquad\qquad U$$
$$G \qquad (X)_a \qquad\qquad\qquad G$$
$$X \qquad * \qquad X \qquad\qquad A \qquad A$$
$$(X)_m \qquad * \qquad (X)_{m'} \qquad G-X$$
$$(X)_b$$

wherein each X represents a ribonucleotide which is the same or different and may be modified or substituted in its sugar, phosphate or base;

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wherein each x represents a deoxyribonucleotide which is the same or different and may be modified or substituted in its sugar, phosphate or base;

wherein each of A, C, U, and G represents a ribonucleotide which may be unmodified or modified or substituted in its sugar, phosphate or base;

wherein each of $(x)_n$ and $(x)_{n'}$ represents an oligodeoxyribonucleotide having a predetermined sequence and each of n and n' represents an integer which defines the number of deoxyribonucleotides in the oligonucleotide with the proviso that the sum of n + n' is sufficient to allow the compound to hybridize with the RNA target sequence;

wherein each * represents base pairing between the nucleotides located on either side thereof;

wherein each solid line represents a chemical linkage providing covalent bonds between the ribonucleotides located on either side thereof;

wherein a represents an integer which defines a number of ribonucleotides with the proviso that a may be 0 or 1 and if 0, the A located 5' of $(X)_a$ is bonded to the X located 3' of $(X)_a$;

wherein each of m and m' represents an integer which is greater than or equal to 1;

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wherein $(X)_b$ represents an oligoribonucleotide and
b represents an integer which is greater than or
equal to 2.

20.   The compound of claim 18, wherein each of m and m' are
one and b is four.

21.   The compound of claim 18, wherein each of m and m' are
seven and b is four.

22.   A compound having the structure:

$$3' \quad [-(Y)_r\text{---}Q\text{---}(Y)_s-]_z \quad 5'$$
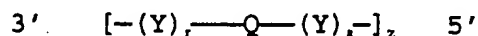
wherein Q represents a compound of claim 18
which may be the same or different;

wherein each Y represents a ribonucleotide
or a deoxyribonucleotide which is the same
or different and may be modified or
substituted in its sugar, phosphate or base;

wherein each of r and s represents an
integer which is greater than or equal to 0;
and

wherein z represents an integer from 1 to
100.

23.   A pharmaceutical composition comprising an amount of
the compound of claim 18 in association with a
pharmaceutically acceptable carrier or excipient.

24.   A method of cleavage of a specific RNA target sequence
which comprises reacting the compound of claim 18 with

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the target sequence so as to thereby cleave the
specific target sequence.

25.   The method of claim 24, wherein the target sequence is
       indigenous to a mammal.

26.   The method of claim 24, wherein the target sequence is
       indigenous to a plant.

27.   The method of claim 24, wherein the target sequence is
       in a viral gene.

28.   A method for the treatment of a viral disease in
       mammals which comprises administering to a mammal an
       effective amount of the compound of claim 18 capable of
       cleaving the viral gene so as to thereby cleave the
       viral gene and treat the viral disease.

29.   The method of claim 28, wherein the virus is a human
       virus such as papillomavirus, hepatitis virus, Epstein-
       Barr virus, human T cell leukemia virus, HIV, herpes
       virus, cytomegolovirus, influenza virus, varicella-
       zoster virus, respiratory syncytial virus, rhinovirus,
       or picornovirus.

30.   The method of claim 28, wherein the administration is
       systemic.

31.   The method of claim 28, wherein the adminstration is
       topical.

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32. A compound having the structure:

$$3' \quad (X)_n\text{——}A \qquad\qquad X\text{——}(X)_{n'} \quad 5'$$
$$A \qquad\qquad\qquad C$$
$$A \qquad\qquad\qquad\quad U$$
$$| \qquad\qquad\qquad\qquad\quad G$$
$$| \qquad\qquad\qquad\qquad\qquad A$$
$$G \qquad\qquad A\text{—}G\text{—}N$$
$$L$$

wherein each X is the same or different and
represents   a   ribonucleotide   or   a
deoxyribonucleotide which may be modified or
substituted in its sugar, phosphate or base;

wherein $(X)_n$ and $(X)_{n'}$ represent oligonucleotides
in which n and n' are integers which define the
number of nucleotides in the oligonucleotides,
such   oligonucleotides   having   predetermined
sequences   sufficiently   complementary   to   a
predefined RNA target sequence to be cleaved to
allow hybridization to the RNA target sequence;

wherein N may be adenine, guanine, cytosine or
uracil;

wherein each solid line represents a chemical
linkage providing covalent bonds between the
nucleotides located on either side thereof;

wherein L represents a linker molecule which may
be a polyether such as polyphosphopropanediol,
polyethyleneglycol,   a   bifunctional   polycyclic
molecule such as a bifunctional pentalene,
indene,   naphthalene,   azulene,   heptalene,
biphenylene,   asym-indacene,   sym-indacene,
acenaphthylene,   fluorene,   phenalene,

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phenanthrene, anthracene, fluoranthene, acephenathrylene, aceanthrylene, triphenylene, pyrene, chrysene, naphthacene, thianthrene, isobenzofuran, chromene, xanthene, phenoxathiin, indolizine, isoindole, 3-H-indole, indole, 1-H-indazole, 4-H-quinolizine, isoquinoline, quinoline, phthalazine, naphthyridine, quinoxaline, quinazoline, cinnoline, pteridine, 4-αH-carbazole, carbazole, ß-carboline, phenanthridine, acridine, perimidine, phenanthroline, phenazine, phenolthiazine, phenoxazine, which polycyclic compound may be substituted or modified, or a combination of the polyethers and the polycyclic molecules.

33. The compound of claim 32, wherein the linker is tetraphosphopropanediol.

34. The compound of claim 32, wherein the linker is pentaphosphopropanediol.

35. The compound of claim 32, wherein the linker is a bifunctional carbazole.

36. The compound of claim 32, wherein the linker is a bifunctional carbazole linked to one or more polyphosphoropropanediol.

37. A compound having the structure:

$$3' \quad [-(Y)_r\!\!-\!\!Q\!\!-\!\!(Y)_s-]_z \quad 5'$$
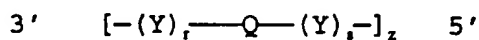
wherein Q represents a compound of claim 32 which may be the same or different;

wherein each Y represents a ribonucleotide or a deoxyribonucleotide which is the same or different and may be modified or substituted in its sugar, phosphate or base;

wherein each of r and s represents an integer which is greater than or equal to 0; and

wherein z represents an integer from 1 to 100.

38. A pharmaceutical composition comprising an amount of the compound of claim 32 in association with a pharmaceutically acceptable carrier or excipient.

39. A method of cleavage of a specific RNA target sequence which comprises reacting the compound of claim 32 with the target sequence so as to thereby cleave the specific target sequence.

40. The method of claim 39, wherein the target sequence is indigenous to a mammal.

41. The method of claim 39, wherein the target sequence is indigenous to a plant.

42. The method of claim 39, wherein the target sequence is in a viral gene.

43. A method for the treatment of a viral disease in mammals which comprises administering to a mammal an effective amount of the compound of claim 32 capable of cleaving the viral gene so as to thereby cleave the viral gene and treat the viral disease.

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44. The method of claim 43, wherein the virus is a human
virus such as papillomavirus, hepatitis virus, Epstein-
Barr virus, human T cell leukemia virus, HIV, herpes
virus, cytomegolovirus, influenza virus, varicella-
5   zoster virus, respiratory syncytial virus, rhinovirus,
or picornovirus.

45. The method of claim 43, wherein the administration is
systemic.
10
46. The method of claim 43, wherein the adminstration is
topical.

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FIG. 1

FIG. 2

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FIG. 3

FIG. 4

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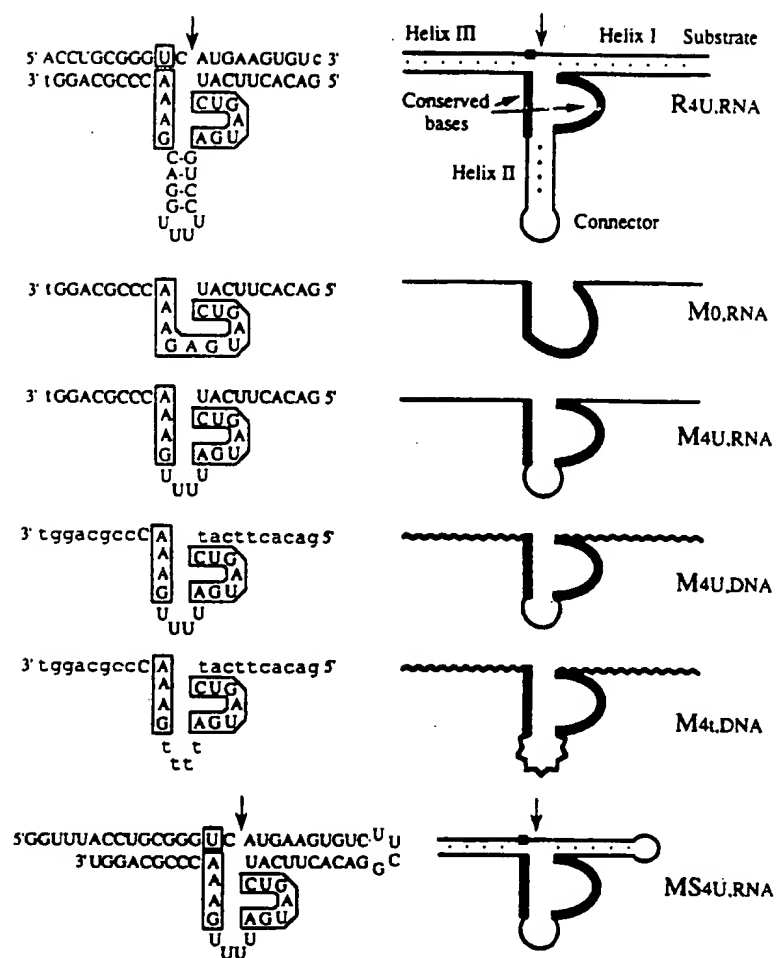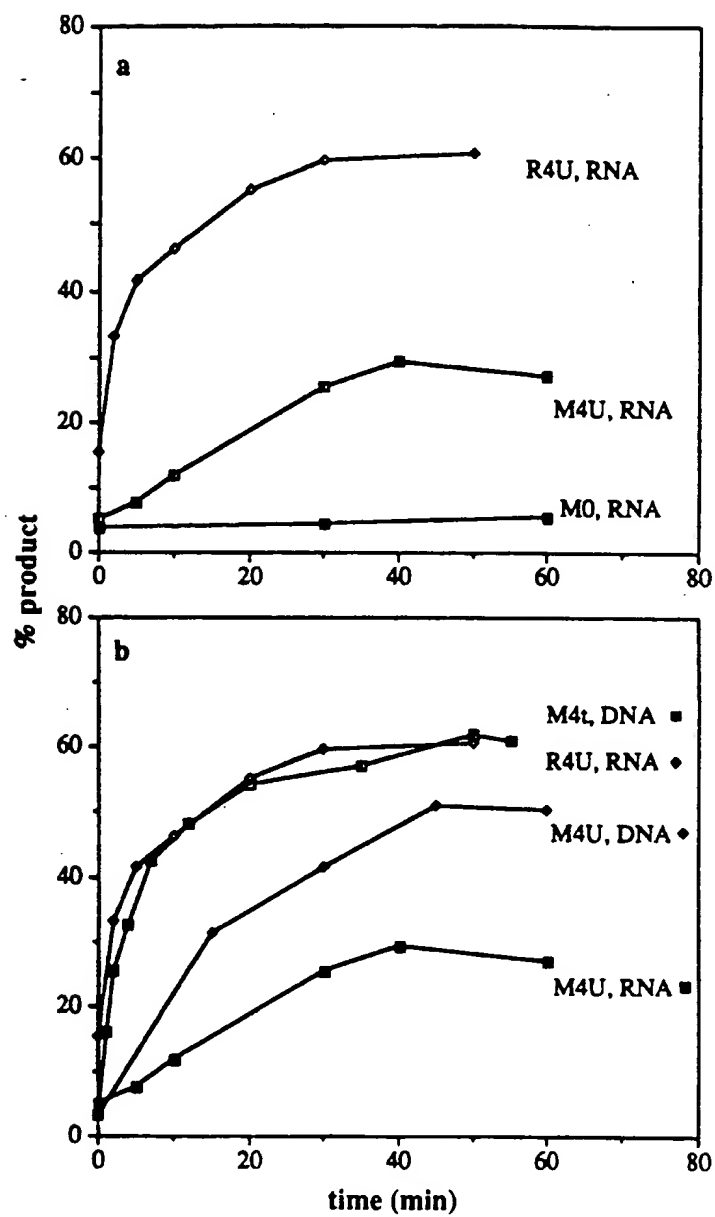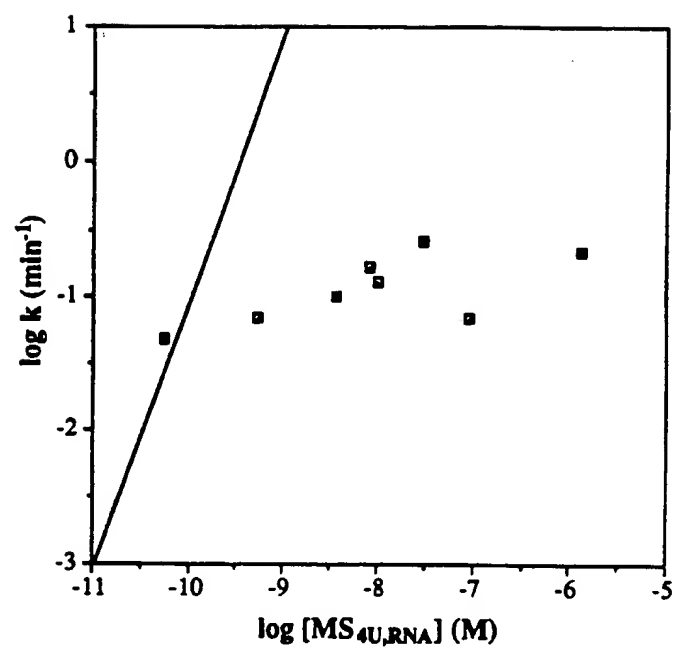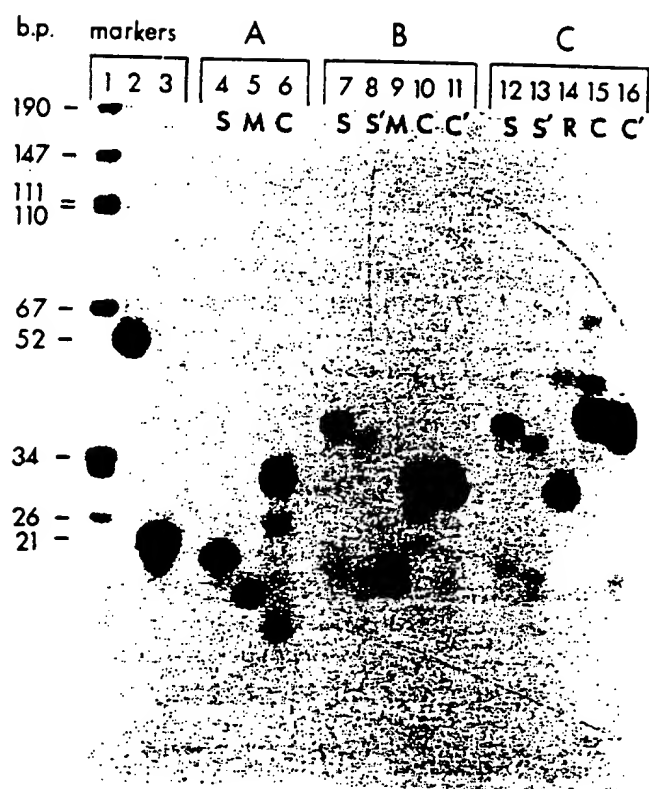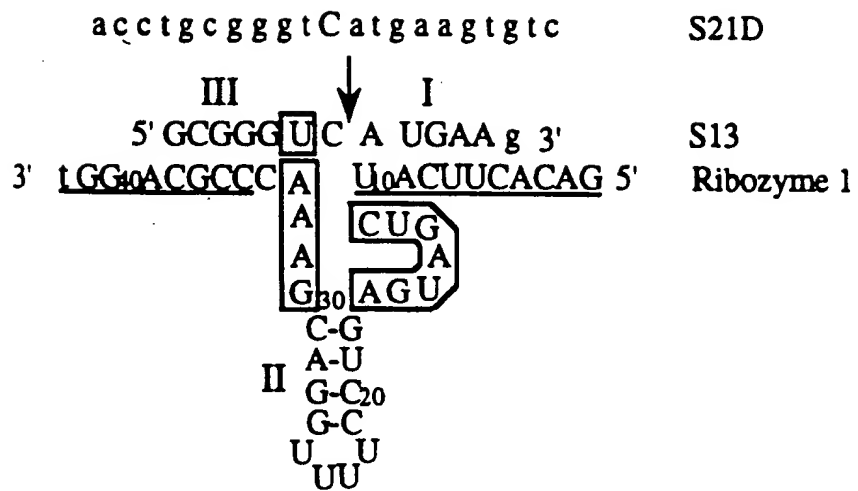
FIG. 5
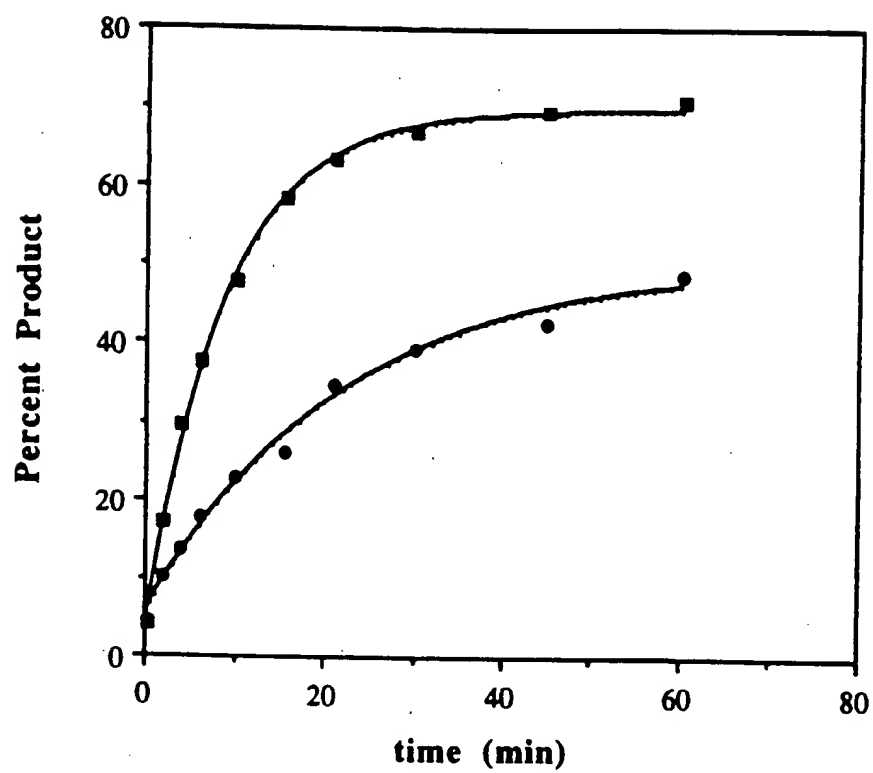
FIG. 6
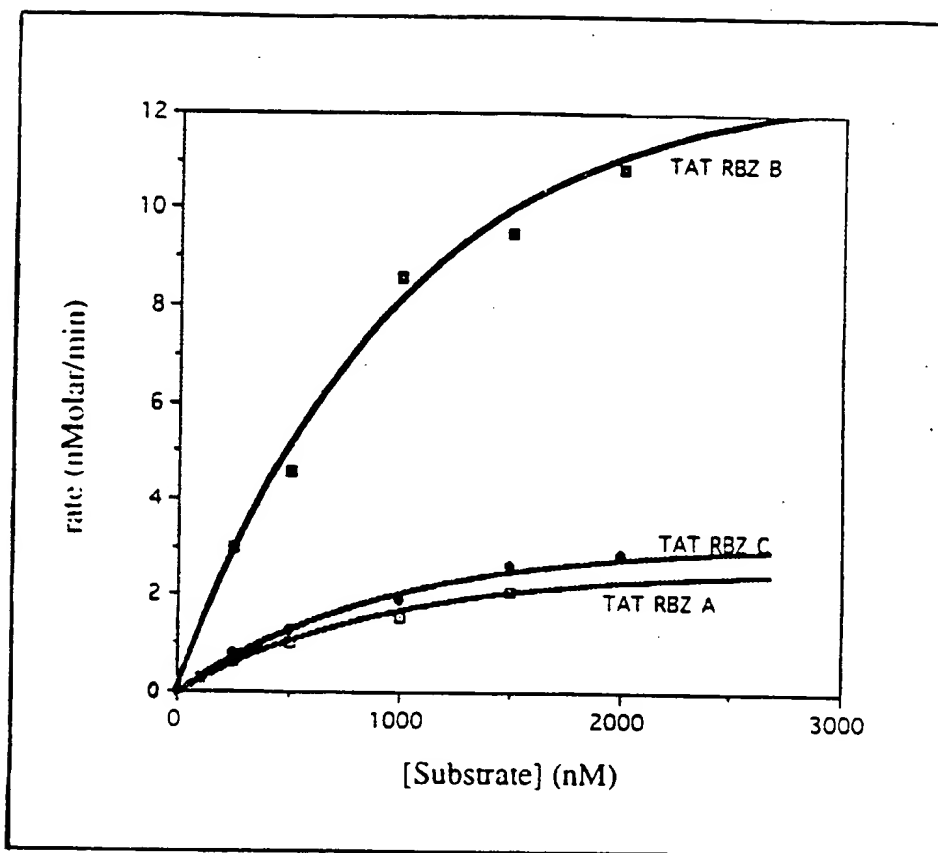
FIGURE 8-1

21-MER SYNTHETIC SUBSTRATE
37°C

▭	TAT5RC
▭	TAT5MD
◇	TAT5ME
▪	TAT5RA
○	TAT5RB
▲	CONTROL
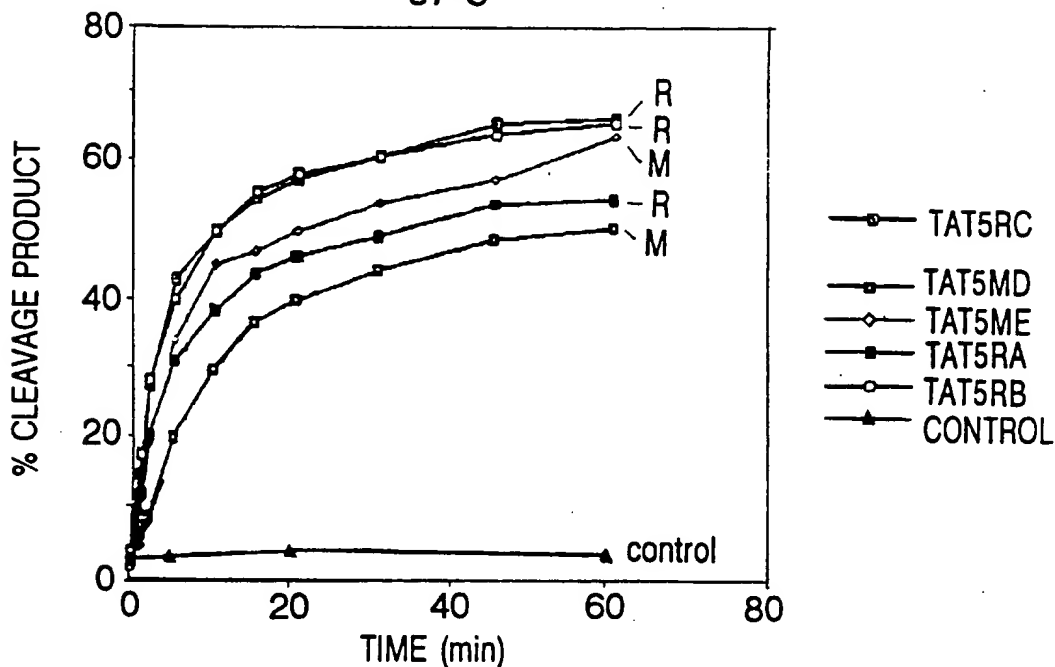
FIGURE 8-2

428 NT TRANSCRIPT
37°C

Concentrations of ribozymes and minizymes are greater than concentrations of substrates in all experiments.

▭	TAT5ME
●	TAT5MD
▭	TAT5RA
○	TAT5RB
▪	TAT5RC
▲	CONTROL

FIG. 9

FIG. 10

```
3' a g g t c c t t C      t c g g a t c c t g  5'
              A    CUG
              A       A
              A    GU
              G    A
               C.G
               G.C
               U   U
                UU
```
TATRB-H2

```
3' a g g t c c t t C      t c g g a t c c t g  5'
              A    CUG
              A       A
              A    GU
              G    A
               C.G
               A.U
               G.C
               G.C
               U   U
                UU
```
TATRB-H4
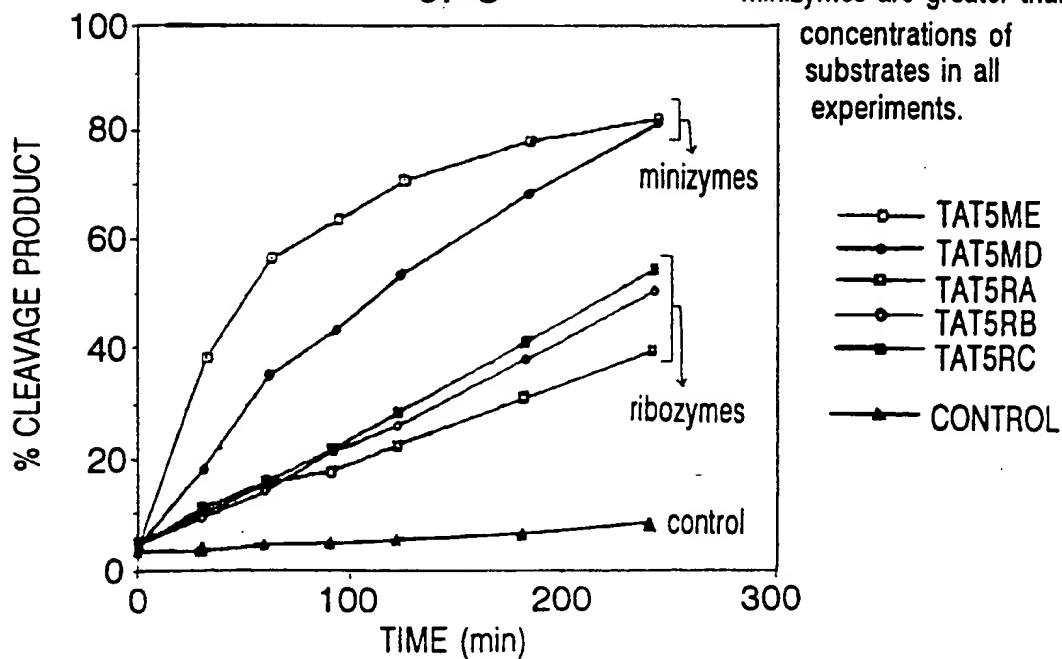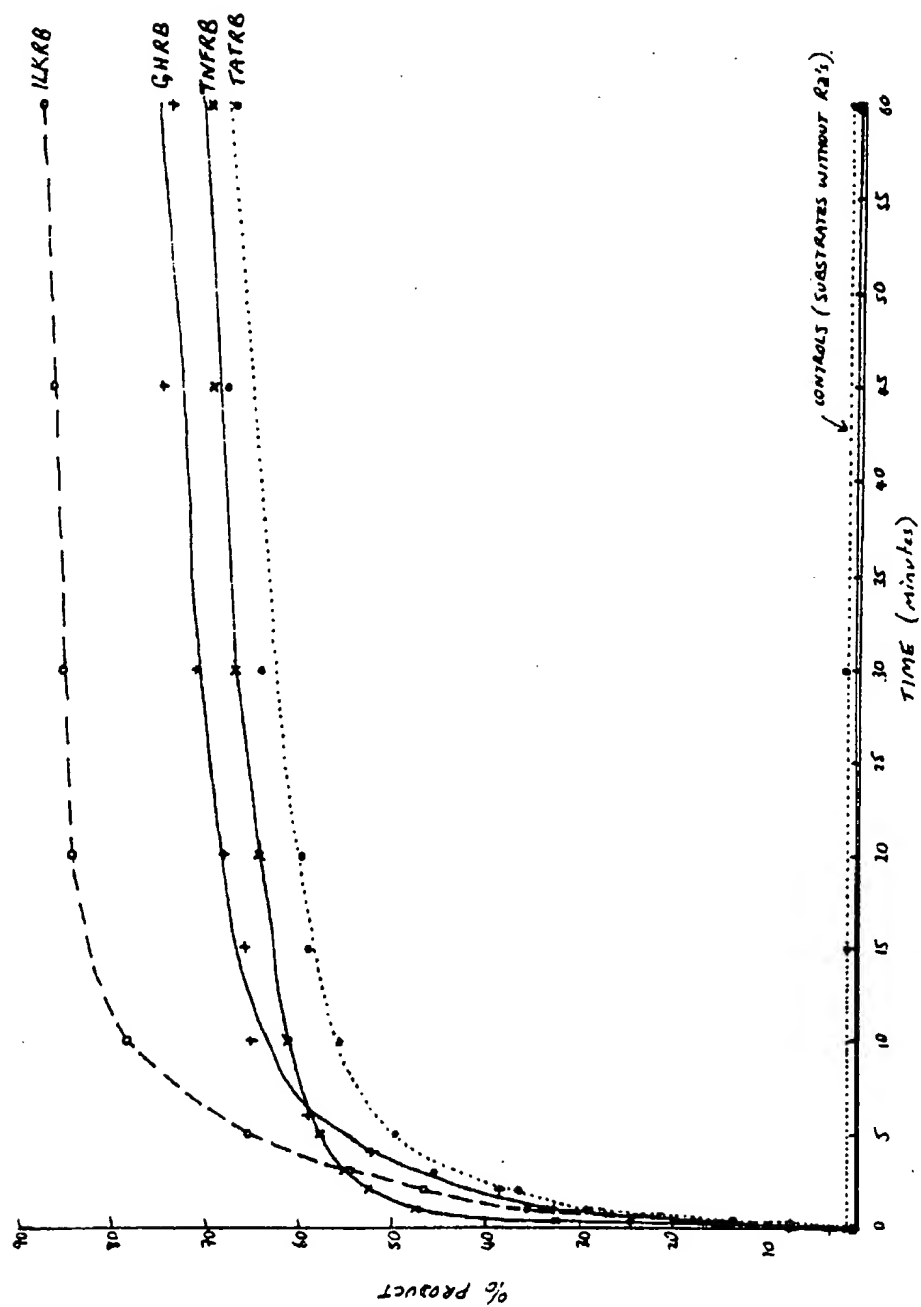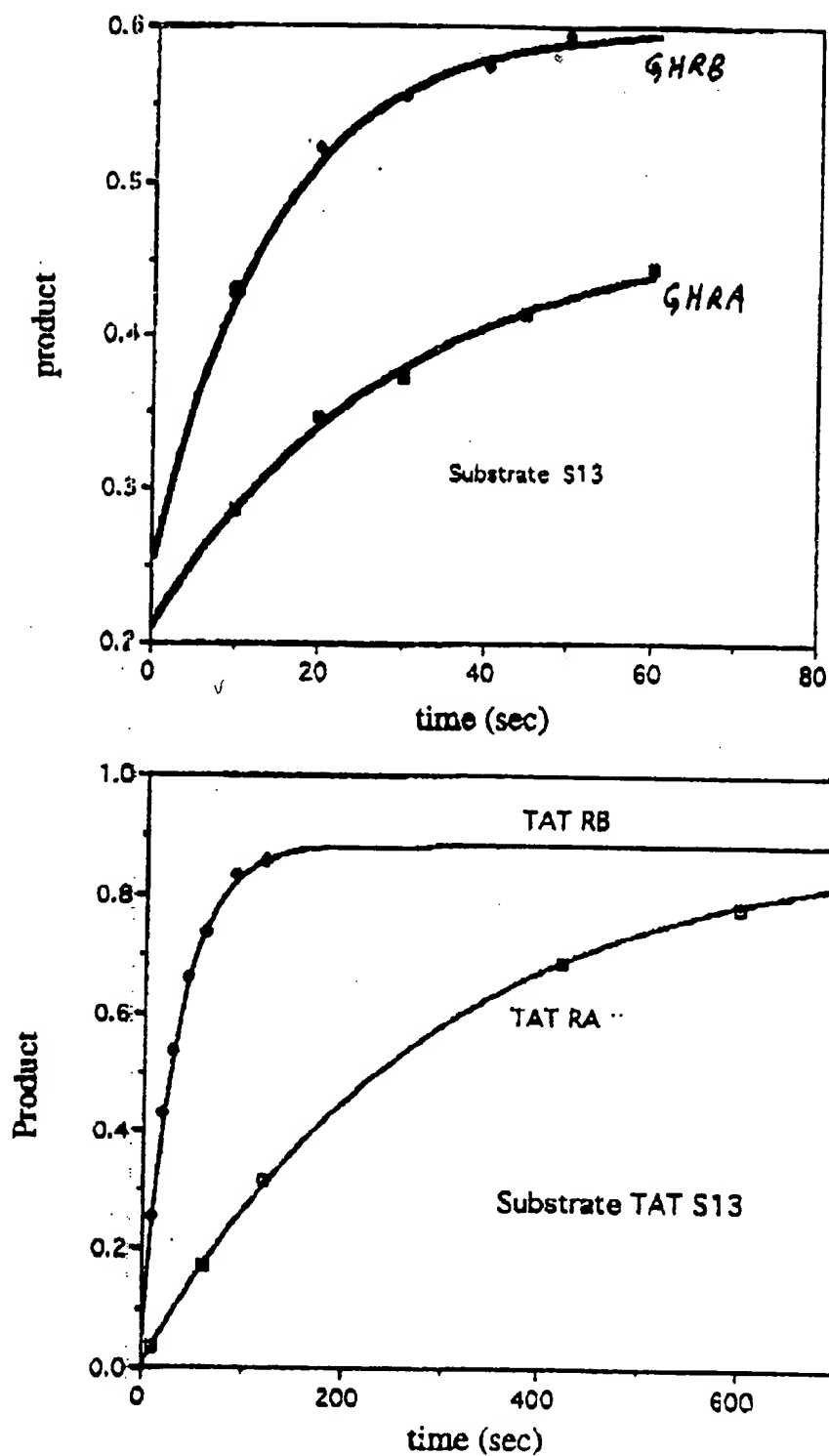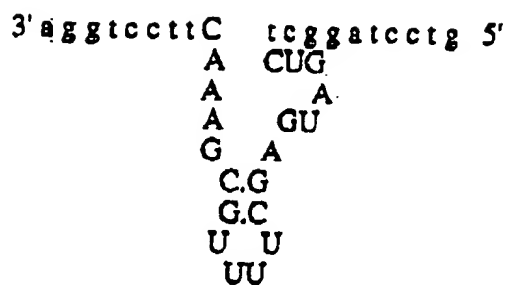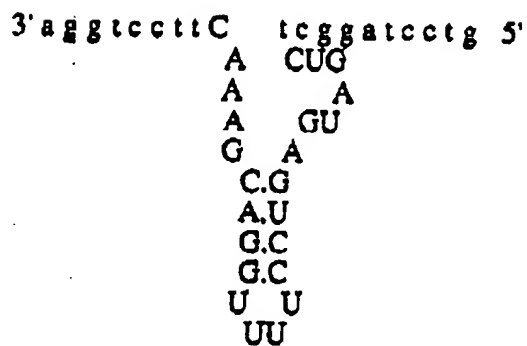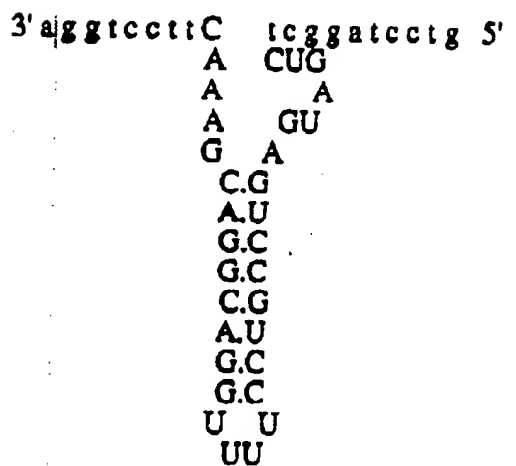
```
3' a g g t c c t t C      t c g g a t c c t g  5'
              A    CUG
              A       A
              A    GU
              G    A
               C.G
               A.U
               G.C
               G.C
               C.G
               A.U
               G.C
               G.C
               U   U
                UU
```
TATRB-H8

FIG. 11

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FIG. 12

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FIG. 13

```
3'        aggtccttCA      tcggatcctg  5'
                 A      CUG
                 A           A
                 G      AGU
                    C-G
                    A-U
                    G-C
                    G-C
                    U   U
                     UU
```
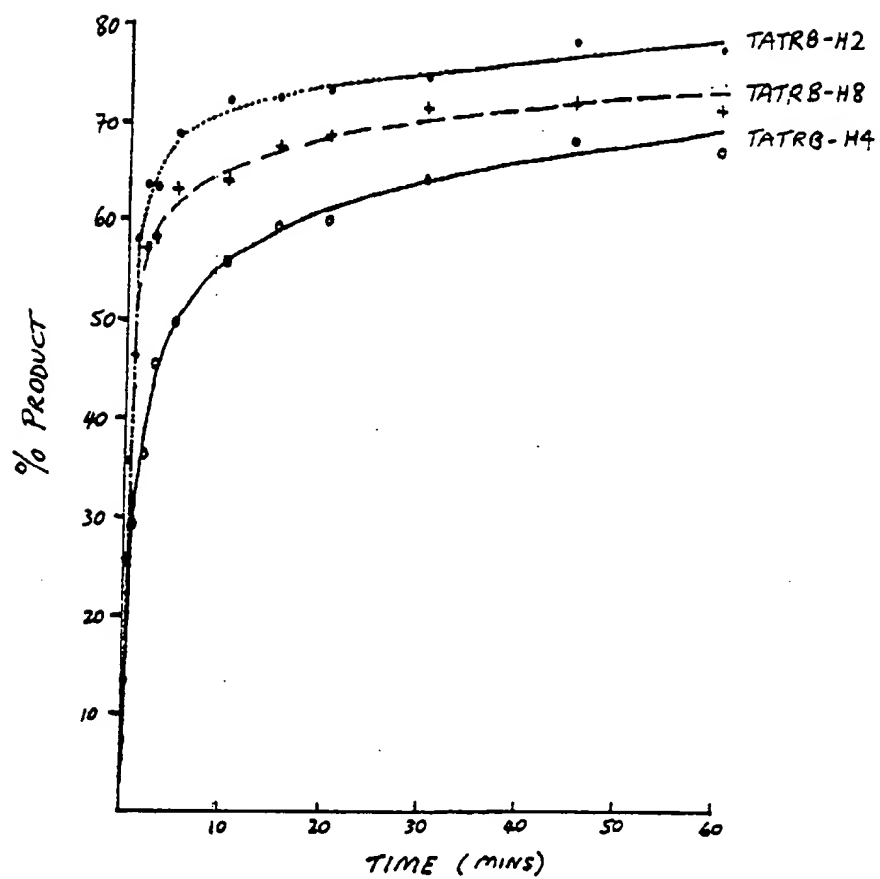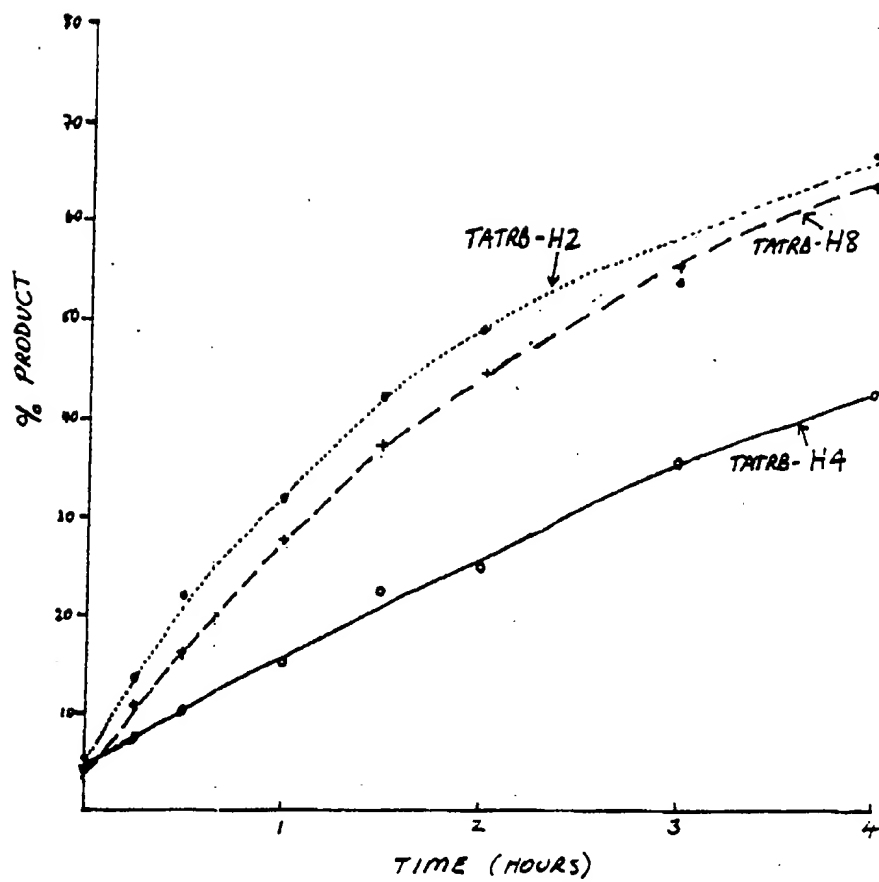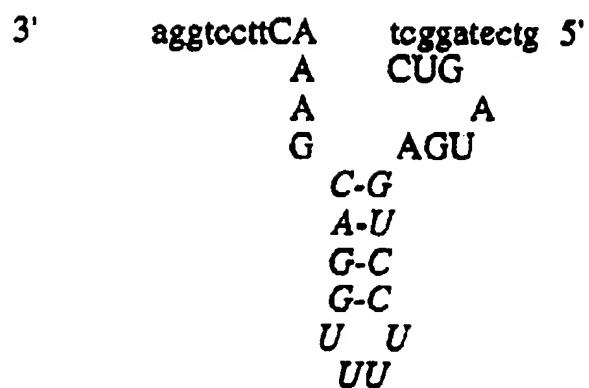
TAT RG (RNA arms and DNA helix II)
```
3' AGGUCCUUCA        UCGGAUCCUG 5'
              A      CUG
              A           A
              G      AGU
                 c-g
                 a-t
                 c-g
                 c-g
                 t   t
                  tt
```
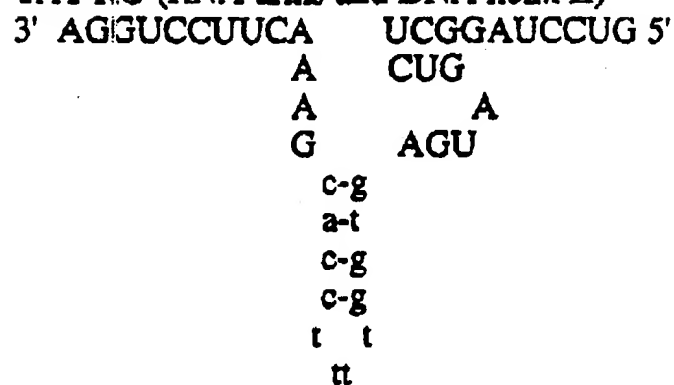
TAT RH (mixture of DNA and RNA in arms and helix II/linker)
```
3'      aggtcCUUCA      UCGGAtcctg 5'
                 A      CUG
                 A           A
                 G      AGU
                    C-G
                    A-U
                    G-C
                    G-C
                    t   t
                     tt
```
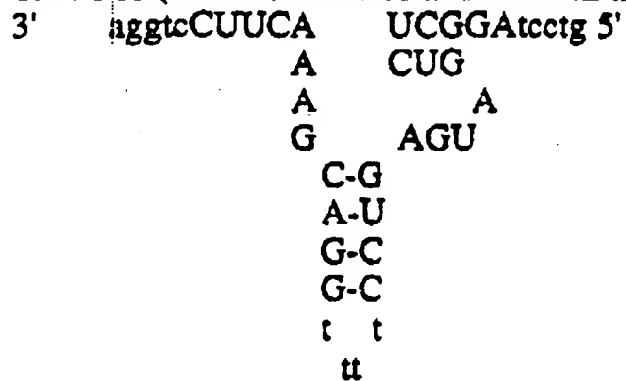
FIG. 14

FIG. 15
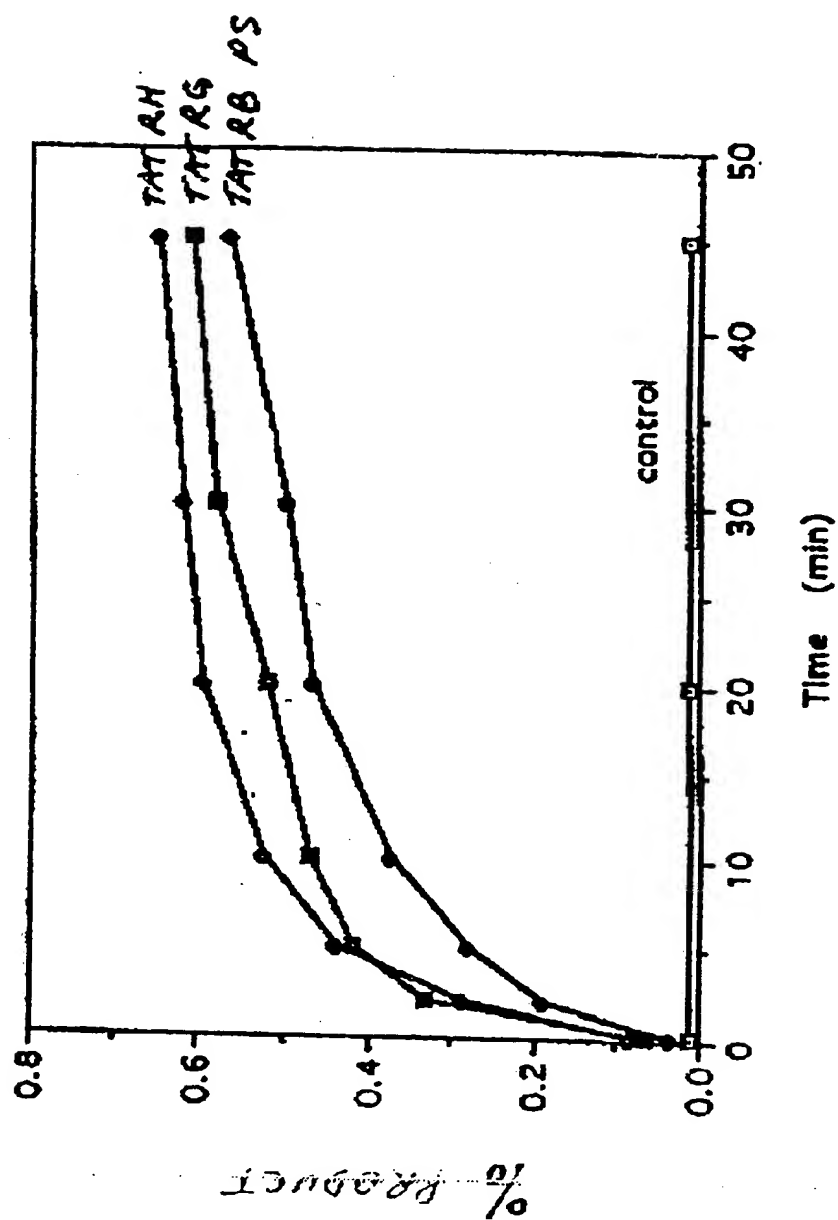
FIGURE 16A

KrS21   5' AUUUGCGAGUCCACACUGGAg 3'

KrM½A   GUGUGACCUC 5'
                                     CUG
                                        A
                                 3' A^{9}GU

KrM½B   3' UAAACGCUCA
                                    A
                                    A
                                    G^{12} 5'

S13   5' GCGGGUCAUGAAg 3'

M(n)pd   3' c g c c CA   t a c t t c 5'
                                    A   CUG
                                    A   A
                                    G   AGU
                                     (pd)$_n$
                                            n = 1,2,3,4,5

FIGURE 16B

Meg

$$3'\; c\; g\; c\; c\; CA \quad t\; a\; c\; t\; t\; c\; 5'$$
$$\qquad\qquad A \quad CUG$$
$$\qquad\qquad A \qquad\quad A$$
$$\qquad\qquad G \quad AGU$$
$$\qquad\qquad\quad eg$$

$$eg = teg,\; heg$$

M(n)t

$$3'\; t\; g\; g\; a\; c\; g\; c\; c\; CA \quad t\; a\; c\; t\; t\; c\; a\; c\; a\; g\; 5'$$
$$\qquad\qquad\qquad A \quad CUG$$
$$\qquad\qquad\qquad A \qquad\quad A$$
$$\qquad\qquad\qquad G \quad AGU$$
$$\qquad\qquad\qquad\; t_n$$

$$n = 2,3,4,5,6$$

M4t(6x2)

$$3'\; c\; g\; c\; c\; CA \quad t\; a\; c\; t\; t\; c\; 5'$$
$$\qquad\qquad A \quad CUG$$
$$\qquad\qquad A \qquad\quad A$$
$$\qquad\qquad G \quad AGU$$
$$\qquad\qquad\; t\quad t$$
$$\qquad\qquad\; tt$$

M_{UUCG}

$$3'\; t\; g\; g\; a\; c\; g\; c\; c\; CA \quad t\; a\; c\; t\; t\; c\; a\; c\; a\; g\; 5'$$
$$\qquad\qquad\qquad A \quad CUG$$
$$\qquad\qquad\qquad A \qquad\quad A$$
$$\qquad\qquad\qquad G \quad AGU$$
$$\qquad\qquad\qquad G \quad U$$
$$\qquad\qquad\qquad C \quad U$$

FIGURE 16C

```
GHRB     3' tggacgccCA tacttcacag 5'
                    A   CUG
                    A      A
                    G   AGU
                    C·G
                    A·U
                    G·C
                    G·C
                    U   U
                    U   U
```
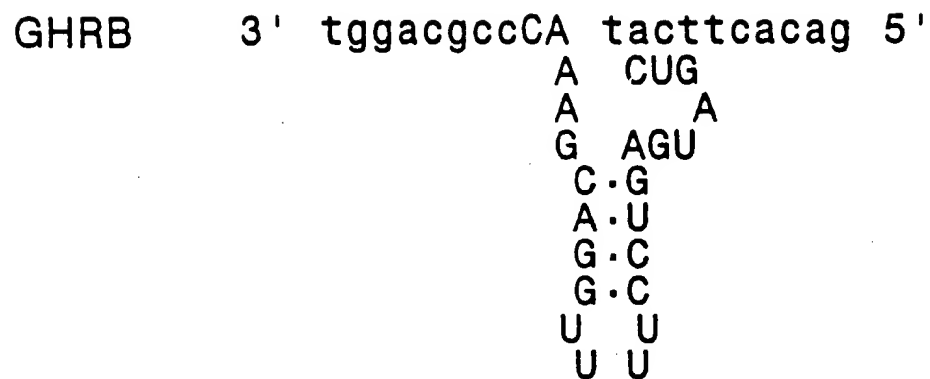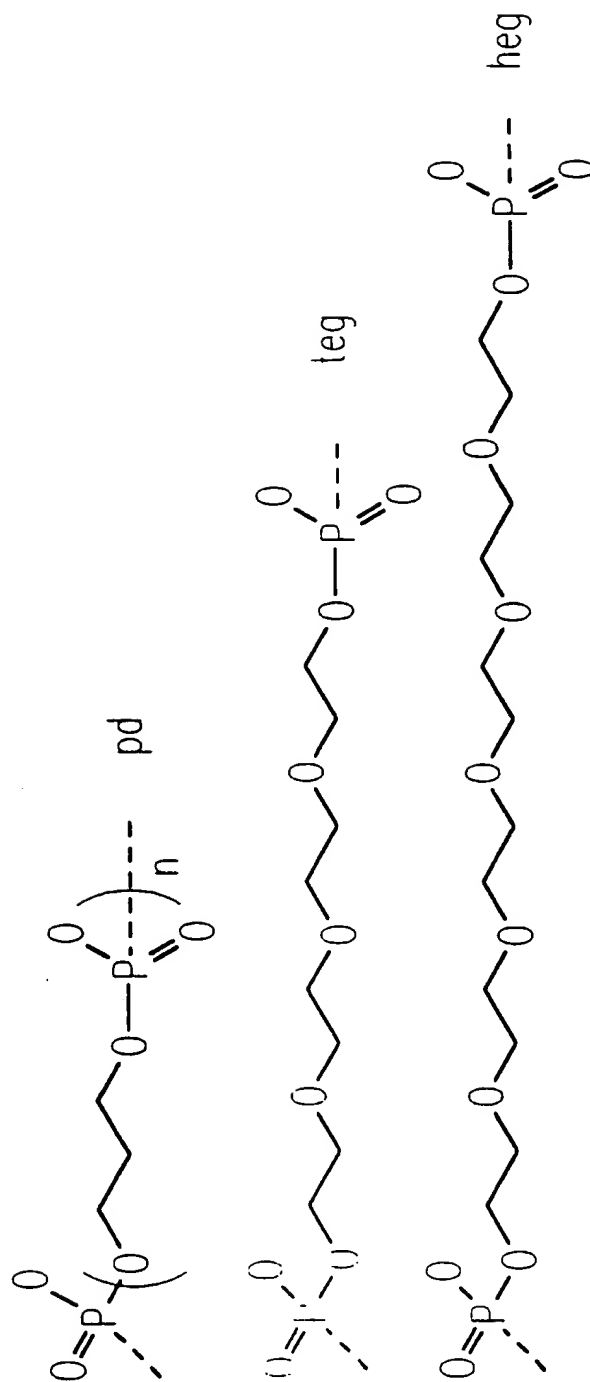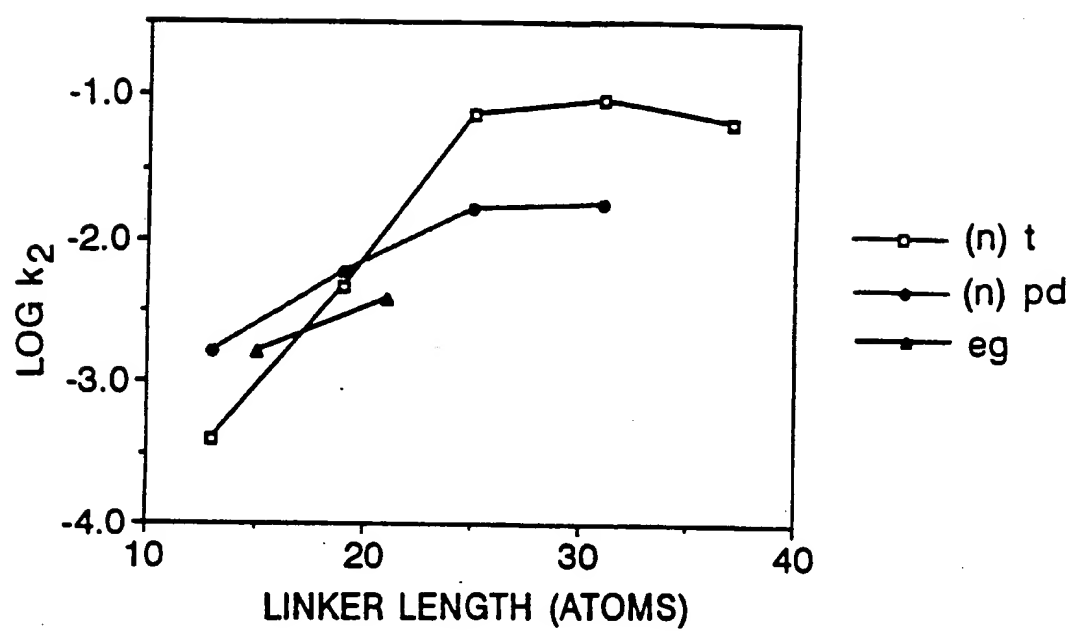
FIGURE 17
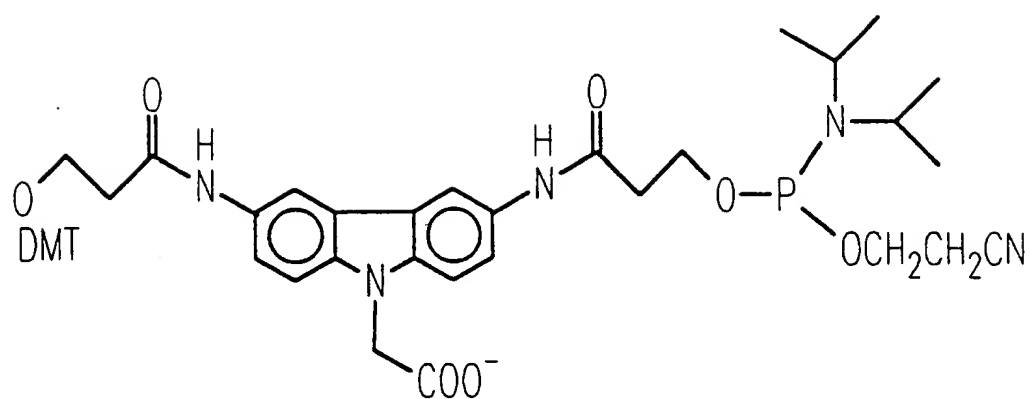
FIGURE 18

FIGURE 19

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FIGURE 20

GH M Carb
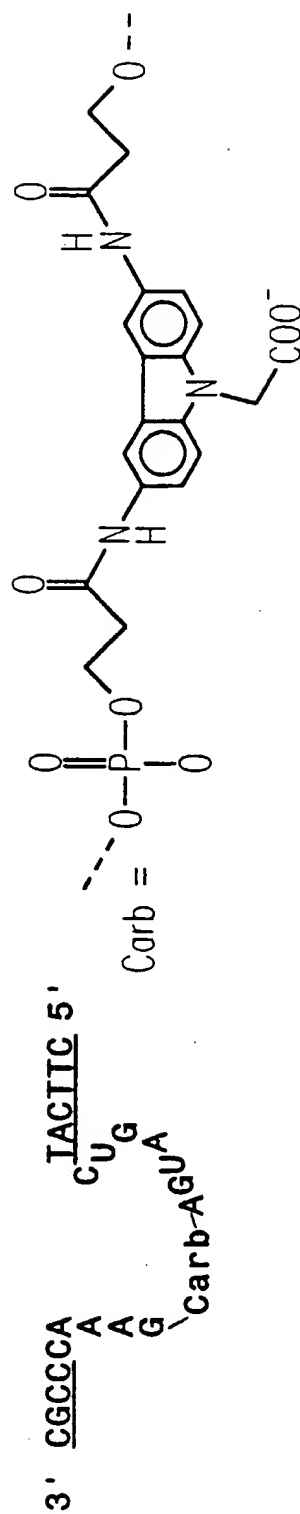
UNDERLINED = DNA

GH M pcp
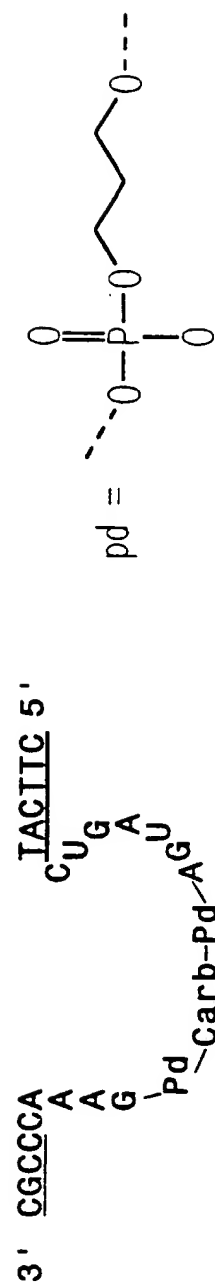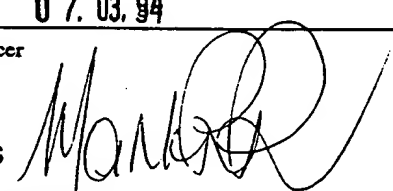
FIGURE 21

CLEAVAGE OF GHS13 BY GH M CARB & GH M PCP

A.	**CLASSIFICATION OF SUBJECT MATTER**

Int. Cl.[5] C07H 21/02, C07H 21/04, C12N 5/10, C12N 15/10, C12N 15/63

According to International Patent Classification (IPC) or to both national classification and IPC

B.	**FIELDS SEARCHED**

Minimum documentation searched (classification system followed by classification symbols)
IPC:C12N 15/10, C12N 15/63, C07H 21/02, C07H 21/04
CHEMICAL ABSTRACTS : SEE BELOW

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base, and where practicable, search terms used)
DERWENT:FILE WPAT:RIBOZYME, MINIZYME, HAMMERHEAD
CHEMICAL ABSTRACTS:FILE CASM:FIGURE 16 SEQUENCES

C.	**DOCUMENTS CONSIDERED TO BE RELEVANT**

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to Claim No.
X Y	AU,A,79976/91 (Commonwealth Scientific and Industrial Research Organisation) Published 26 December 1991 (26.12.91). See abstract, Example 2 (Page 30) in particular.	1-31,33-34,36-46 32,35
X Y	AU,B,58177/90 (City of Hope) Published 8 April 1991 (08.04.91). See abstract, claims 1-8, figures 1-2.	1-2,4-5,7,12-13,15-21,23-25, 27-29 3,6,8-11,14,22,26,30-46

X	Further documents are listed in the continuation of Box C.		X	See patent family annex.

*	Special categories of cited documents :	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance		
"E"	earlier document but published on or after the international filing date	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)		
"O"	document referring to an oral disclosure, use, exhibition or other means	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"P"	document published prior to the international filing date but later than the priority date claimed		
		"&"	document member of the same patent family

Date of the actual completion of the international search 24 January 1994 (24.01.94)	Date of mailing of the international search report 0 7. 03. 94
Name and mailing address of the ISA/AU AUSTRALIAN INDUSTRIAL PROPERTY ORGANISATION PO BOX 200 WODEN ACT 2606 AUSTRALIA	Authorized officer MARK ROSS
Facsimile No. 06 2853929	Telephone No. (06) 2832295

C(Continuation).	DOCUMENTS CONSIDERED TO BE RELEVANT	
Category*	Citation of document, with indication, where appropriate of the relevant passages	Relevant to Claim No.
X Y	McCall, M.J., Hendry, P. and Jennings, P.A. (1992) "Minimal Sequence Requirements for Ribozyme Activity", Proceedings of the National Academy oif Sciences, USA, vol.89, pp 5710-5714, Published July 1992. See figure 1 in particular.	1-5,18-21 6-16,22-31
X Y	Hendry, P., McCall, M.J., Santiago, F.S., and Jennings, P.A. (1992) "A Ribozyme with DNA in the Hybridising arms displays enhanced cleavage ability", Nucleic Acids Research, vol. 20, pp 5737-5741, published November 11, 1992. See figure 1 in particular.	1-5,18-21 6-16,22-31

This Annex lists the known "A" publication level patent family members relating to the patent documents cited in the above-mentioned international search report. The Australian Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent Document Cited in Search Report		Patent Family Member						
AU	79976/91	CA	2084790	EP	535067	FI	925715	
		IL	98543	NO	924861	NZ	238614	
		WO	9119789	ZA	9104652			
AU	58177/90	CA	2039716	DE	4091533	EP	451221	
		GB	2242432	US	5149796	WO	9103162	
		WO	9206988	US	5272262	WO	9100213	
		EP	478713					

END OF ANNEX